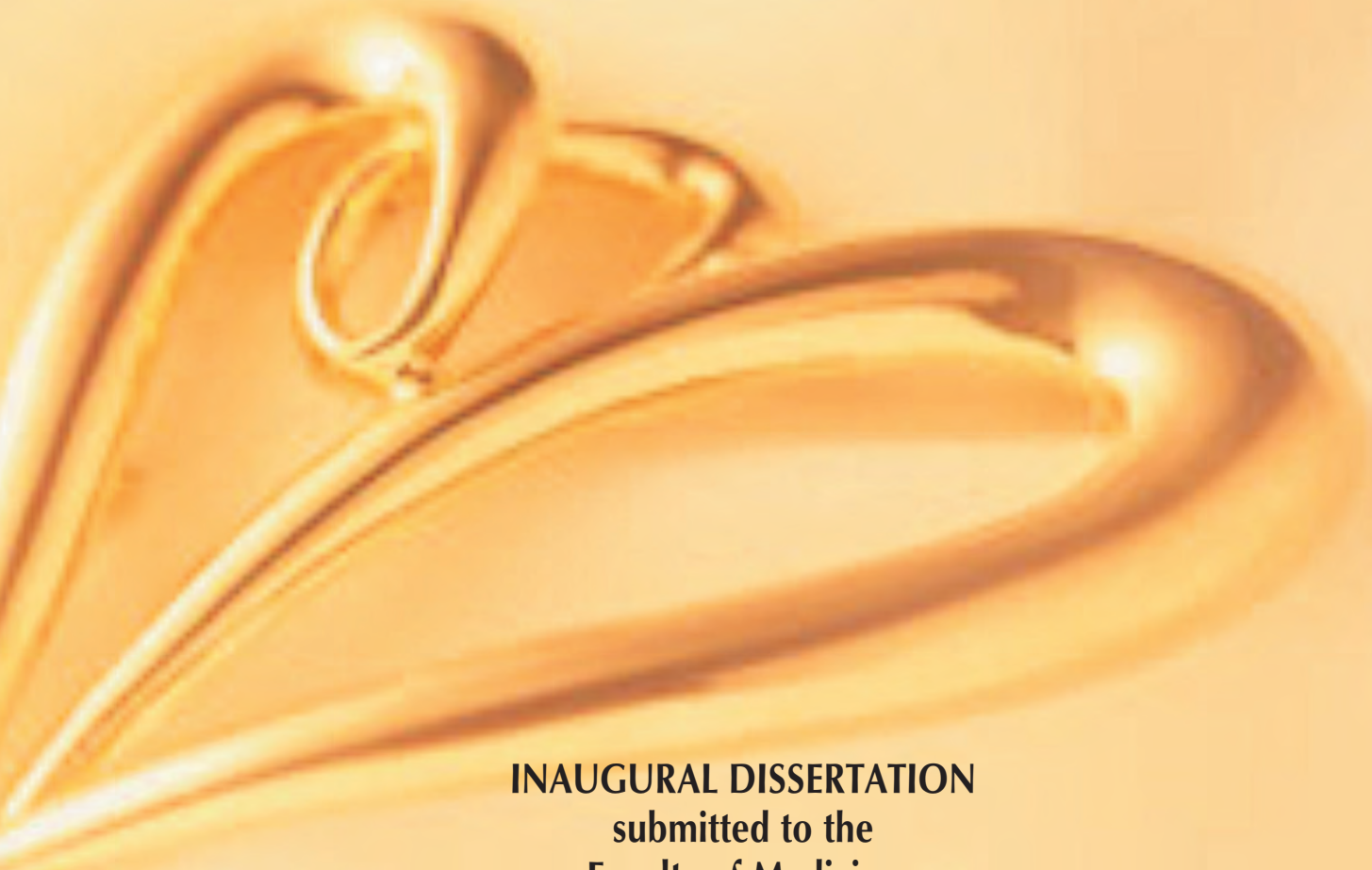
FUNCTIONAL ROLE OF NFAT IN VENTRICULAR CARDIOMYOCYTES OF RAT

ATTIA ANWAR



INAUGURAL DISSERTATION
submitted to the
Faculty of Medicine
in partial fulfillment of the requirements
for the PhD-Degree
of the Faculties of Veterinary Medicine and Medicine
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by

Attia Anwar

of

Gujrat-Pakistan

Giessen 2006

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Date of Doctoral Defense: October 09th, 2006

Dedicated to

My Parents

Who have always worked very hard for making the best available education accessible to me. I thank them for always believing in me and always being there for me. This thesis is truly a fruit of their lifelong affection and prayers.

&

My Sweet Husband

Whose constant encouragement and somewhat prolonged banishment together provided me incentive and opportunity to complete the compilation of this thesis.

Table of contents

Abbreviations

% (vol/vol)	Volume percent
% (wt/vol)	Weight volume percent
AA/BAA	Acrylamide /Bisacrylamide
AKAP79	A-kinase anchoring protein 79
AP	Alkaline phosphatase
AP-1	Activator protein-1
APS	Ammonium persulphate
BAPTA/AM	1,2-(Bis (2-aminophenoxy) ethane-N,N,N´,N´-tetra acetic-acid tetrakis (acetoxy methyl-ester))
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate toluidine salt
BDM	2,3-butanedion monoxime
bp	Base pair
BPB	Bromophenolblue
BSA	Bovine serum-albumin
C	Control
Ca^{2+}	Calcium
Ci	Curie, Unit for the radioactivity
Cyclo	Cyclosporine
DEPC	Diethyl dicarbonate
DNA	Deoxyribonucleic acid
dNTP's	deoxy nucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylen diamine tetra acetic acid
FCS	Fetal calf serum

Gö6850	2-(1-(3-Dimethylaminopropyl)-1H-indol-3-yl)-3-(1H-indol-3-yl)-maleimide
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethanesulphonic acid
HPRT	Hypoxanthin phosphoribosyl transferase
Hz	Frequency in cycles per second
ISO	Isoprenaline
MCIP1	Modulatory calcineurin-intracting protein 1
MEF2	Myocyte enhancer-binding factor-2
n	Numbers
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
Na-Vanadat	Sodium vanadat
NBT	Nitro blue tetrazolium chloride
NCX	Sodium calcium exchanger
NFAT	Nuclear factor of activated T-cells
NHE	Sodium proton exchanger
OD	Optical density
ODC	ornithine dicarboxylase
p	Error probability
PBS	Phosphate buffered saline
PE	Phenylephrine
PI3-kinase	Phosphatidyle inositole 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Phospholamban

PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidine difluoride
RACK	Receptor for activated C kinase
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcrition polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SERCA	Sarcoplasmic reticulum calcium ATPase
SR	Sarcoplasmic reticulum
TAE	Tris-acetate/EDTA-buffer
TE	Tris/HCl/EDTA-buffer
TEMED	N,N,N,N-Tetra methyl ethylene diamine
Tm	Melting temperature
Tris	Tris (hdroxylmethyl)-amino-methane

1. Introduction

1.1. Myocardial hypertrophy and heart failure

Myocardial hypertrophy represents an adaptation of cardiomyocytes to overload in the heart. This can be caused either by increased pressure or increased volume and results in an enhanced work load. To compensate this increased demand, wall thickness of the heart increases. This is accomplished by an increase in protein synthesis, so that cell volume increases and wall thickness is enhanced without an increase in the number of cardiomyocytes.

Although hypertrophic growth is a physiological answer to increasing work load, it can finally result in heart failure. An example of a cause of increased work load is chronically arterial hypertonus. In this situation the heart is able to adjust the pressure gradient between ventricles and aorta by an increase in wall thickness. By the increase of the diameter of muscle fiber the wall tension can be kept constant. If continuous pressure overload persists, the increased myocardial volume reaches the critical heart weight, and the heart muscle mass exceeds the capacity of coronary arteries to supply the heart sufficiently with oxygen. At the same time a rise in wall tension as well as a structural dilation of the ventricle occurs which reduces efficiency of heart function. Thus, myocardial hypertrophy has to be regarded as a starting point for development of chronic heart failure with increased morbidity (Levy et al., 1990). Understanding of mechanisms resulting in hypertrophy and functional impairments in hypertrophic myocytes are therefore an important step towards therapy of heart disease.

1.2. α-Adrenoceptor stimulation induces hypertrophy

Increased plasma levels of catecholamines are commonly found under conditions leading to myocardial hypertrophy in vivo (Bugaisky et al., 1992; Morgan et al., 1991). This gave rise to the question whether induction of hypertrophy by adrenergic agonists represents a direct metabolic effect on cardiomyocytes or if it is the result of an altered mechanical response. Although the adaptation of the heart to pressure overload is considered as an adaptive process, myocardial hypertrophy is a predictor of heart failure (Levy et al., 1990).

Multiple stimuli are able to increase protein synthesis of cardiomyocytes (for an overview see ref. Schlüter & Wollert, 2004). However, in the early adaptation of the heart to pressure overload catecholamines seem to play a major role. In fact, hearts from transgenic mice deficient in β-dopamine hydroxylase do not respond adequately in terms of hypertrophic growth when they are challenged with pressure overload (Rapacciulo et al., 2001). Moreover, transgenic mice lacking α_1-adrenoceptors and challenged with pressure overload had impaired survival rates (O'Connell et al., 2003). These results suggest that catecholamines via stimulation of α-adrenoceptors are responsible for adaptive processes of myocardial hypertrophy due to pressure overload.

Isolated cardiomyocytes, which are quiescent under culture conditions, have been used as an experimental model to clarify this point. Stimulation of α-adrenoceptors in isolated, cultured, non-beating cardiomyocytes from adult animals causes hypertrophy (Decker et al., 1993; Fuller et al., 1990; Schlüter and Piper, 1992). This response is characterized by an increase in protein mass of the cells, increase in RNA synthesis (Clark et al., 1993; Decker et al., 1993; Schlüter and Piper, 1992) and an increase in cell size (Clark et al., 1993). No marked differences occur between different species: similar results have been identified in cardiomyocytes obtained from adult rats (Fuller et al., 1990; Schlüter and Piper, 1992), adult rabbits (Decker et al., 1993) and adult cats (Clark et al., 1993).

From work with isolated and cultured cardiomyocytes, proteins that are specifically up regulated under conditions of cardiac hypertrophy can be placed in three categories:

1) Expression of transcription factors.
2) Expression of structural proteins.
3) Extracellular signaling factors.

Most of the signal transduction pathways of hypertrophic growth under α-adrenoceptor stimulation are protein kinase C (PKC) dependent. Action of α-adrenergic agonists increases protein kinase C activity (Schlüter et.al., 1995). PKCε activation causes a physiologic form of hypertrophy, whereas inhibition of

PKCε translocation with a RACK-binding peptide causes the opposite response, that is, thinning of the ventricular walls and lethal heart failure in the form of a dilated cardiomyopathy (Mochly-Rosen et al., 2000). These studies suggest that PKCε activation is a necessary component of normal trophic growth of cardiomyocytes during postnatal development.

Under α-adrenoceptor stimulation PKC activates PI3-kinase that results in increased protein and RNA synthesis (Schlüter et al., 1998; Pinson et al., 1993). Because >90% of total RNA consists of rRNA, this finding indicates an increase in the capacity of cardiomyocytes to synthesize proteins. In neonatal cardiomyocytes it has been shown that the transcription of rDNA can be activated in a protein kinase C-dependent way (Allo et al., 1992).

Protein kinase C and PI 3-kinase as well as the $p70^{s6}$-kinase increase the protein synthesis by α-adrenoceptor stimulation in adult cardiomyocytes (Pönicke et al., 2001). It has been reported that in neonatal cardiomyocytes activation of $p70^{s6}$-kinase is also involved in the hypertrophic growth response to α-adrenoceptor agonists (Boluyt et al., 1997).

Besides the PKC-dependent signaling, also PKC-independent pathways contribute to hypertrophic growth (Schäfer et al., 2002), i.e. a PKC-independent activation of the sodium-proton-exchanger (NHE) is found after α-adrenoceptor stimulation of cardiomyocytes (Schäfer et al., 2002). This NHE activation is mediated by increases in cytosolic Ca^{2+} induced by the α-adrenoceptor agonist phenylephrine (PE). Activation of NHE by phenylephrine causes cytosolic alkalization (Fuller et al., 1991; Schlüter et al., 1998). This leads to an increase in the creatine phosphate concentration due to alterations of the creatine kinase equilibrium. In whole hearts and isolated cardiomyocytes a correlation between protein synthesis and creatine phosphate concentration has been observed (Fuller et al., 1989). In isolated cardiomyocytes a contribution of NHE to hypertrophic growth was shown, which is in part dependent on creatine phosphate (Schlüter et al., 1999)

1.2.1. Calcium-calcineurin-NFAT-AP-1 signaling

In response to myocyte stretch or increased loads on working heart preparations, intracellular Ca^{2+} concentration increases (Marban et al., 1987; Bustamante et al., 1991; Hongo et al., 1995), consistent with a role of Ca^{2+} in coordinating physiologic responses with enhanced cardiac output. A variety of humoral factors, including phenylephrine (PE), which induce the hypertrophic response in cardiomyocytes (Karliner et al., 1990; Sadoshima and Izumo, 1993; Sadoshima et al., 1993; Leite et al., 1994), also share the ability to elevate intracellular Ca^{2+} concentrations. In addition, as stated before, hypertrophic growth of PE-induced cardiomyocytes is in part mediated via activation of NHE by Ca^{2+}. Another effect of Ca^{2+} is activation of the phosphatase calcineurin, which is responsible for activation of the transcription factor NFAT (Rao et al., 1997).

Molkentin et al. (1998) and De Windt et al (2000) generated several lines of transgenic mice expressing activated forms of either calcineurin or NFATc4 in a cardiac-selective manner, which developed robust hypertrophy that quickly transitioned to ventricular dilation and overt heart failure. Hearts from transgenic mice expressing MCIP1, a dominant negative calcineurin mutant, or the calcineurin inhibitory domains of Cain or AKAP79, were largely resistant to pleiotropic, hypertrophic stimuli (Zou et al., 2001; Rothermel et al., 2001; De Windt et al., 2001). Adenoviral-mediated gene transfer of dominant negative NFAT in cultured cardiomyocytes efficiently inhibited calcineurin- and agonist induced cardiomyocyte hypertrophy (Rooij et al., 2002). As outlined above until now only under the conditions of unphysiologically high expression of NFAT/calcineurin or their inhibitors in transgenic mice or by adenoviral over-expression have been shown to effect myocardial hypertrophy. Therefore, it remains an open question whether under physiological conditions NFAT influences cardiomyocyte hypertrophy or contractility.

In addition to the transcription factor NFAT, there are several studies indicating also involvement of the transcription factor AP-1 in hypertrophy. Correlations between formation of AP-1 and hypertrophic growth have been shown in several experimental models (Izumi et al., 2000; Yano et al., 1998; Takemoto et al.,

1999) also including phenylephrine (PE)-stimulated neonatal cardiomyocytes (Omura et al., 2002). During α-adrenoceptor stimulation AP-1 is induced and involved in hypertrophic growth in ventricular cardiomyocytes of adult rats (Taimor et.al., 2004). Interestingly, NFAT and AP-1 can interact with each other and therefore may influence their transcriptional activity.

In cells of the immune system, cooperative NFAT-AP-1 complexes are induced by stimulation of the antigen receptors of T and B cells, Fcγ receptors of macrophages and natural killer (NK) cells (Rao et al., 1997). These receptors are coupled on the one hand to calcium mobilization and on the other hand to activation of PKC/RAS pathways (Van Leeuwen and Samelson, 1999). Full response at many NFAT sites requires concomitant activation of members of the AP-1 transcription-factor family (Rao et al., 1997; Jain et al., 1992). Therefore, even in a single cell type NFAT activation can evoke two distinct biological programs of gene expression that depending on AP-1 absence or presence (Macian et al., 2000). It remains an open question whether NFAT contributes to the AP-1 mediated hypertrophic growth in PE induced cardiomyocytes.

1.3. Effect of β-adrenoceptor agonists in hypertrophy

Compared with the hypertrophic action mediated by α_1-adrenoceptor activation, the mechanism by which β-adrenoceptor stimulation may induce hypertrophy in isolated cardiomyocytes is more complicated. There is no evidence that stimulation of β-adrenoceptors directly increases the rate of protein synthesis in freshly isolated cardiomyocytes from adult rats (Bogoyevitch et al., 1993; Pinson et al., 1993; Schlüter and Piper, 1993). There are, however, reports that coupling of β-adrenoceptors to a hypertrophic response can be induced during cultivation in rat cardiomyocytes in the presence of low concentrations of isoprenaline (Dubus et al1990) or in the presence of 20 % fetal calf serum (Pinson et al., 1993). In these cultures, cardiomyocytes release inactive transforming growth factor-β into the medium, which is activated subsequently by serum factors. Active transforming growth factor-β induces the hypertrophic responsiveness to β-adrenoceptor stimulation. This situation mimics the ability of isoprenaline to

induce myocardial hypertrophy in vivo (Bartlome et al., 1980, Schlüter et al., 1995). In all these cases the rate of protein synthesis, cellular protein mass, and cell size increased.

In the case of β-adrenoceptor stimulation, however, RNA elevation is not accompanied by increase in RNA synthesis (Pinson et al., 1993). The mechanism by which β-adrenoceptor stimulation elevates RNA mass seems to be due to a decrease of RNA degradation, which can be mediated by stabilization of RNA by polyamines (Igarashi et al., 1982). Ornithine decarboxylase (ODC) represents the rate-limiting enzyme of polyamine metabolism. ODC is involved in the mechanism by which β-adrenoceptor stimulation elevates cellular RNA mass (Cohen SS., 1998). β-Adrenoceptor-mediated hypertrophy in vivo is also accompanied by induction of ODC (Bartolome et al., 1980).

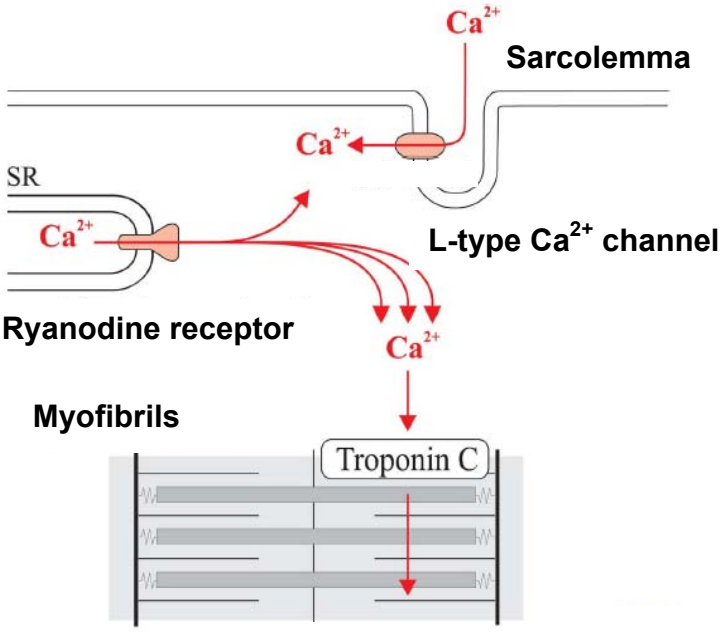
In adult rat cardiomyocytes cAMP-dependent protein kinase-A (PKA) and PI3-kinase as well as $P70^{s6k}$ increase protein synthesis by β-adrenoceptor stimulation in long term cultures (Schlüter et.al., 1998; Simm et.al.,1998). Involvement of the transcription factor AP-1 was excluded in β- adrenoceptor-mediated hypertrophy. (Taimor et al., 2004) Interestingly, AP-1 is induced by β-adrenoceptor stimulation also in freshly isolated cardiomyocytes, a condition that is not related to hypertrophic growth.

1.4. Contractile function of cardiomyocytes

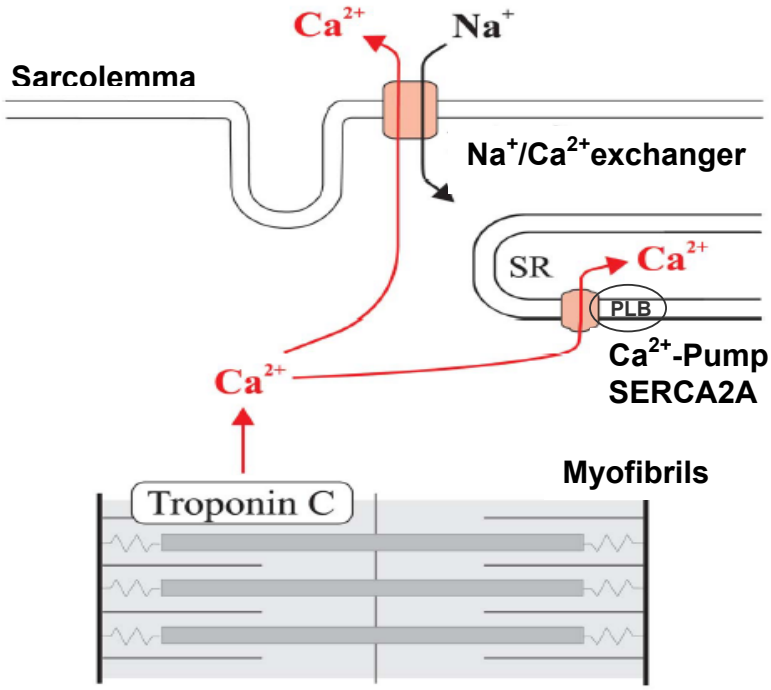
The aforementioned studies have given us some insights how cardiomyocytes respond to catecholamines in regard to hypertrophic growth. However, it has not been investigated whether this leads to an altered contractile function of the cell. A regulated release and uptake of intracellular Ca^{2+} between sarcoplasmic reticulum (SR) and cytoplasm tightly controls the contraction and relaxation cycle of the heart. The Na^{+}-Ca^{2+} exchanger (NCX) is one of the essential regulators of Ca^{2+} homeostasis in cardiac myocytes and plays an important role in Ca^{2+} handling during excitation-contraction (E-C) coupling. The exchanger is capable of transporting 3 Na^{+} for 1 Ca^{2+} in either direction across the sarcolemma,

depending on membrane potential and the transmembrane gradients of Na^+ and Ca^{2+}. The NCX is the primary mechanism for extruding Ca^{2+} that enters through the L-type Ca^{2+} channel during systole. Maintenance of the calcium concentration within the SR is accomplished by calcium buffers, calcium channels and calcium pumps.

Muscle contraction is initiated when Ca^{2+} enters the cell via L-type Ca^{2+} channels in the plasmalemma and as a consequence, triggers the release of a much larger amount of Ca^{2+} from the SR via SR Ca^{2+} release channels (ryanodine receptor) (Fabiato A, 1983; Bers and Perez-Reyes 1999) (Fig: 1.4.1). Cell contraction occurs when calcium ions bind to the troponin/tropomyosin complex, changing its shape so that the binding sites on actin are exposed. The free cytosolic Ca^{2+} concentration determines the extent of the muscle activation and therefore regulates force development. The SR Ca^{2+} ATPase (SERCA) pumps the Ca^{2+} back into the SR and is therefore, responsible for muscle relaxation and for replenishing Ca^{2+} stores needed for the next contraction (MacLennan DH, 1970) (Fig: 4.1.1). SERCA pump activity is regulated by the small, 52-amino acid phosphoprotein phospholamban (PLB), which in its unphosphorylated state lowers the affinity of SERCA for Ca^{2+} (Simmermann and Jones, 1998).



Contraction



Relaxation

Figure: 1.4.1. Depiction of the calcium release and storage in cardiomyocytes. The calcium ions are actively pumped into the SR by SERCA2a, bound in the lumen by calcium buffer, and released into the cytosol by ryanodine receptors. This movement of calcium ions across the SR membrane is necessary for muscle contraction and relaxation.

A number of studies suggest that alterations in SR Ca^{2+} handling are a critical feature of the hypertrophied or failing myocardium. Alterations in the expression of different SR proteins and associated Ca^{2+} transport abnormalities in cardiac hypertrophy and heart failure have been reviewed by Houser et al. (2000). The SERCA2a isoform plays a central role in SR Ca^{2+} handling required for excitation-contraction coupling in the heart. Moreover, it was shown for mouse, rat and rabbit that the expression of SERCA pump gradually increases during development (Luss et al., 1999; Reed et al., 2000; Chen et al., 2000; Fisher et al., 1992; Gombosova et al., 1998). This increase was accompanied by a shortening of relaxation time in neonatal ventricle (Gombosova et al., 1998). In adult heart SERCA levels are not steady but influenced by aging and fluctuations in thyroid hormone level. A decrease in content and activity of SERCA was described in experimental models of senescence and in senescent human myocardium (Taffet et al., 1993; Cain et al., 1998). This decrease was associated with a prolonged contraction time and depressed myocardial function. Therefore, several naturally occurring variations in SERCA expression level correlate with the contractile status of the heart. The expression level of SERCA pump protein appears to be a critical determinant of cardiac contractility.

Varying degrees of defects in the SR Ca^{2+} uptake have been identified in animal models of heart disease and have been shown to correlate with altered contractile function (reviewed in Arai et al., 1994). Studies from many laboratories have shown that the expression level of SERCA is significantly decreased in pressure overload-induced hypertrophy/heart failure (Nagai et al., 1989; Feldman et al., 1993; Matsui et al., 1995; Qi et al., 1997; Aoyagi et al., 1999). In these studies decreased SR calcium transport was observed (Arai et

al., 1994; Feldman et al., 1993; 1993; Matsui et al., 1995; Qi et al., 1997; Kiss et al., 1995). This down-regulation of SERCA2a may affect calcium handling and contribute to contractile dysfunction, as suggested by the improved contractility of hypertrophied myocardium following SERCA2a protein over expression using transgenic approaches (Muller et al., 2003; Meyer and Dillmann, 1998). Over expression of SERCA2a using an adenoviral gene transfer technique transiently enhances cardiac contractile function and SR Ca^{2+} uptake (Giordano et al., 1997; Miyamoto et al., 2000). Enhanced contractility has also been reported in SERCA-over expressing transgenic mice not subjected to pathological stimuli (He et al., 1997; Baker et al., 1998).

The reduction of myocardial SERCA2a mRNA and protein expression in pathological hypertrophy was attributed to reduced SERCA2a promoter activity (Dumas et al., 1997). This finding was further corroborated by lower activity of a -1800 bp SERCA2a promoter fragment when transfected in vivo in pressure-overloaded, hypertrophic hearts (Aoyagi et al., 1999; Takizawa et al., 1999). In previous studies influences of calcineurin on SERCA expression were suggested. In myocardial hypertrophy induced by calcineurin over expression, both a fall in SERCA2a mRNA (Molkentin et al 1998) and a rise in SERCA2a protein (Chu et al., 2002) have been observed. In neonatal cardiomyocytes an up-regulation of SERCA2a mRNA expression was found in absence of contractile activity. This is likely due to calcineurin signaling and synergistic stimulation of the SERCA2a promoter by NFATc4 and MEF2c. Since α-adrenoceptor signaling may activate NFAT via the calcineurin pathway, it is an interesting question whether α-adrenoceptor stimulation can enhance cardiomyocyte contractility via NFAT dependent up-regulation of SERCA.

1.5. Cell culture model

To investigate the influence of Ca^{2+}/calcineurin /NFAT signaling on hypertrophy and contractility, studies should be performed on rat ventricular cardiomyocytes. Culture models for cardiomyocytes exist both for neonatal and for heart muscle cells of adult animals. Adult cardiomyocytes are more relevant for heart disease

since myocardial hypertrophy (heart failure) occurs particularly in adult patients. Therefore, in this work only cardiomyocytes of adult rat were used.

Cultivation of isolated cardiomyocytes permits investigation of myocardial hypertrophy on a cellular level. In defined media the effects of selected neurohumoral factors in well-known active substance concentrations can be examined independent of their hemodynamic effects. This represents an advantage in relation to the conditions in vivo. Since cultivated cardiomyocytes are primarily mechanically inactive, cell culture offers the further advantage of allowing observations of cellular reactions to individual stimuli independent of mechanical influences.

On the other hand, cardiomyocytes can be electrically stimulated at a specific contraction frequency, and thus conditions of the contracting heart can be simulated. Contraction function can be analysed in detail by determination of maximum cell contraction, and the contraction and relaxation velocity. By these methods effects of specific stimuli (adrenergic stimulation) on contractile function can be analysed independent of their hemodynamic effects on the heart. This can be achieved by use of a high frequency detection system with 500 Hz.

1.6. Aims of the study

The aim of this study is to analyse the effects of NFAT signaling on PE-induced hypertrophy in isolated rat cardiomyocytes. Furthermore, influences of long-term exposure (24 h) of cardiomyocytes to α– or β-adrenergic stimulation on contractile function will be analysed.

To investigate this it was determined,

1. if NFAT inhibition by decoy oligonucleotides influences α-adrenoceptor agonist induced hypertrophy.
2. if contractile function is altered in cardiomyocytes after 24 h stimulation by the α-adrenoceptor agonist PE or the β-adrenoceptor agonist ISO.
3. if α- or β-adrenoceptor agonists effect SERCA expression in isolated cardiomyocytes.

4. if calcium/NFAT signaling is involved in SERCA regulation during PE or ISO activation.
5. if calcium/NFAT signaling mediates altered contractile function in PE- or ISO-treated cardiomyocytes.

2. MATERIALS

2.1. Chemicals

^{14}C-phenylalanine	Amersham, Freiburg
Acrylamide	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
APS	Serva, Heidelberg
Ascorbic acid	Fluka, Taufkirchen
BCIP	AppliChem, Darmstadt
Benzonase	Merck, Darmstadt
Bisacrylamide	Roth, Karlsruhe
Bromophenoleblue	Sigma, Taufkirchen
BSA	Roche Diagnostics, Mannheim
Calcium chloride	Merck, Darmstadt
Carbogene	Messer Griesheim, Krefeld
Carnitine	Sigma, Taufkirchen
Collagenase, Type CLS II	Biochrom, Berlin
Creatine	Sigma, Taufkirchen
Cy3-dcTP-dye	Amersham Bioscience, Freiburg
Cytosin-β-Arabinofuranoside	Sigma, Taufkirchen
Dithiothreitol	Sigma, Taufkirchen
DNAse-free RNAse	Qiagen, Hilden
Ethanol	Merck Bioscience, Darmstadt
Ethidium bromide	Sigma, Taufkirchen
Fetal calf serum	PAA, Cölbe
Gentamycin	Invitrogen, Karlsruhe
Glacial acetic acid	Merck Bioscience, Darmstadt
Glucose	Sigma, Taufkirchen
HCl	Merck, Darmstadt
HEPES	Invitrogen, Karlsruhe
Hoechst 33258 (Hoe 33258)	Sigma, Taufkirchen

Human activin A	R&D systems, Wiesbaden
Isoprenaline	Sigma, Taufkirchen
Isopropanol	Roth, Karlsruhe
Magnesium Chloride Hexa hydrate	Fluka, Taufkirchen
Magnesiumsulphate	Merck, Darmstadt
Medium 199/ Earl's Salts	Biochrom, Berlin
Mercaptopropandiol	Merck, Darmstadt
Methanol	Merck, Darmstadt
Myostatin	R&D systems, Wiesbaden
Natrium chloride	Merck, Darmstadt
Natriumvanadat	Merck, Darmstadt
Nitrobluetetrazolium	AppliChem, Darmstadt
Penicillin-Streptomycin	Invitrogen,Karlsruhe
Phenylmethylsulfonylfluorid	Sigma, Taufkirchen
Phenylephrine	Sigma, Taufkirchen
poly-(dIdC)	Roche Diagnostics, Mannheim
Potassium Chloride	Merck, Darmstadt
Propidiumiodide	Sigma, Taufkirchen
Proteinase K	Merk Bioscience
Sodium dodecyl sulphate	Merck Bioscience, Darmstadt
Sodium hydroxide	Roth, Karlsruhe
Sucrose	Merck, Darmstadt
Taurine	Sigma, Taufkirchen
TEMED	Roth, Karlsruhe
Terminal Transferase	New England Biolabs, Frankfurt am Main
Trichloroacetic acid	Merck Bioscience, Darmstadt
Tris base	Roth, Karlsruhe
Tris/HCl	Roth, Karlsruhe
Triton X-100	Serva, Heidelberg
TRIzol Reagent	Invitrogen, Karlsruhe

Tween20	AppliChem, Darmstadt
Xylene cyanol	Sigma, Taufkirchen

2.1.1. Inhibitors

BAPTA	Calbiochem, Schwalbach
Gö6850	Calbiochem, Schwalbach
Cyclosporine	Fluka, Taufkirchen

2.2. Decoy oligonucleotides

NFAT	Invitrogen, Karlsruhe
Mut-NFAT	Invitrogen, Karlsruhe

2.3. Antibodies

SERCA (C-20)	Santa Cruz Biotechnology, USA
Bovine antigoat	Santa Cruz Biotechnology, USA
Anti-Actin	Sigma, USA
Anti-Rabbit	Sigma, USA

2.4. RT-PCR reagents

RNAse inhibitor	Promega, USA
DNAse	Invitrogen, Karlsruhe
0.1 M DTT	Invitrogen, Karlsruhe
50mM $MgCl_2$	Invitrogen, Karlsruhe
MMLV-RT	Invitrogen, Karlsruhe
5XRT-buffer	Invitrogen, Karlsruhe
Oligo dt	Roche, Mannheim
dNTP's	Invitrogen, Karlsruhe
10XPCR-buffer	Invitrogen, Karlsruhe
Taq DNA polymerase	Invitrogen, Karlsruhe
100 bp-DNA Ladder	New England Biolabs, Frankfurt a.M.

2.4.1. Real-time RT-PCR Kit

ABsolute SYBR Green Fluorescein Mix	ABgene, Hamburg

2.4.2. Primers

β-actin (forward and reverse)	Invitrogen, Karlsruhe
HPRT (forward and reverse)	Invitrogen, Karlsruhe
SERCA2A (forward and reverse)	Invitrogen, Karlsruhe

All other chemicals used in this work were obtained with the best analytical quality by the following companies: Invitrogen (Karlsruhe), Amersham-Bioscience (Freiburg), Merck (Darmstadt) and Sigma (Taufkirchen).

All chemicals were dissolved and stored regarding the manufacturers instruction. For the preparation of solutions, media and buffers millipore water was used, if not mentioned differently.

2.5. Equipments

2.5.1. General objects of utility

Gel electrophoresis chamber	Biotec Fisher, Reiskirchen
Retardation gel chamber	Amersham Bioscience, Freiburg
Western blot apparatus	Biotec Fisher, Reiskirchen
Centrifuge (Type: 18)	Kendro, Hanau
Glass ware	Schott, Mainz
Magnetic stirrer with hot plate	Jahnke & Kunkel, Staufen
pH meter	WTW, Weilheim
Pipettes	Eppendorf-Netheler-Hinz, Hamburg
System for the production of distilled water	Millipore, Eschborn
Thermo cycler	Techne, Wertheim-Bestenheid
Waterbath (Julabo U3)	Julabo Labortechnik GmbH, Seelbach

Gel documentation system	INTAS, Göttingen

2.5.2. Special objects of utility

2.5.2.1.Cell culture

Dissection instruments	Aeskulap, Heidelberg
Incubator (Cytoperm)	Kendro, Hanau
Langendorff-Apparatus	University, Giessen
Nylon net (Pore size 200 µm)	Neolab, Heidelberg
Microscope (TMS-F)	Nikon, Japan
Sterile bench (Lamin Air® HBB2472)	Kendro, Hanau
Cell scraper	Becton Dickinson, Heidelberg
Tissue chopper	Harvard Apparatus, March-Hugstetten

2.5.2.2. Other instruments

Liquid scintillation counter	Canberra-packard, Frankfurt a.M.
Microtiter plate photometer	Dynatech, Denkendorf
Photometer	Amersham, Freiburg
Scintillation container	Canberra-packard, Frankfurt a.M.
Fluo-imager	Bio.Rad Laboratories, München
Icycler	Bio.Rad Laboratories, München

2.5.3. System for the measurement of cell contraction parameters

Interface INT4	Scientific Instruments GmbH, Heidelberg
Microscope	TMS-F Nikon, Japan
Monitor	Philips
One-dimensional camera ZK4	Scientific Instruments GmbH, Heidelberg
Oscillograph	Scientific Instruments GmbH,

	Heidelberg
Stimulator	Physiology lab of institute of Physiology, JLU, Giessen

2.5.4. Consumables

Culture dishes (Falcon 3001 -3004)	Becton Dickinson, Heidelberg
Gloves	NOBA pvt. Wetter
Pipette-Tips	Sarstedt, Nümbrecht
Reaction tubes (0.5/ 1.5/ 2.0 ml)	Eppendorf-Netheler-Hinz, Hamburg

2.5.5. Software for analysis

Image-Quant	Molecular Dynamics, Krefeld
MUCEL	Scientific Instruments GmbH, Heidelberg
Excel	Microsoft
SPSS	SAS software-Version 6.11

3. METHODS

3.1. Isolation of ventricular cardiomyocytes

3.1.1. Laboratory Animals

Ventricular cardiomyocytes were isolated from two- to three- month-old male Wistar rats. These 300- to 350-g male rats were bred in the animal house of the Institute of Physiology at the Justus Liebig University, Giessen.

3.1.2. Preparation of isolated ventricular cardiomyocytes from rat hearts (Piper et al., 1982)

The following solutions were used for the preparation of cardiomyocytes.

Calcium-stock solution:

$CaCl_2$	100 mM

Powell-Medium: (Carbogen gassed):

NaCl	110 mM
$NaHCO_3$	25 mM
Glucose	11 mM
KCl	2.6 mM
KH_2PO_4	1.2 mM
$Mg_2SO_4 \times H_2O$	1.2 mM

Collagenase buffer:

Powell-Medium	40 ml
Collagenase	25 mg
Calcium Stock solution	12.5 µl

3.1.3. Procedure for the preparation of cardiomyocytes

At the beginning of the preparation, the Langendorff perfusion system was flushed with Powell medium and then filled bubble-free with 80 ml Powell medium and warmed up to 37°C. To obtain a constant pH value, Powell medium was gassed with carbogen throughout the whole preparation. Wistar rats (appr. 300 g) were anaesthetized for 1-2 min with diethyl ether. The chest was opened and the diaphragm was dissected. The hearts together with the lungs were transferred into a large Petri dish containing ice-cold salt solution (0.9 % NaCl). Lung, esophagus, trachea and thymus were removed. The flow of perfusion was started with 1 drop per sec. The hearts were mounted on cannula of the Langendorff apparatus by slipping the aorta over the cannula. The heart was fixed with a clamp. The appended heart was flushed blood free with 40 ml Powell medium and then retrograde perfuse with 50 ml recirculating collagenase buffer.

After perfusion aorta and atria were removed and ventricles were chopped in pieces with a tissue chopper (slitting width 0.7 mm). Chopped tissue was digested with 30 ml carbogen-gassed collagenase buffer for 5 min at 37 °C. To separate single cells, the suspension was pipetted up and down several times with a sterile 5 ml pipette. The material was filtered through a nylon mesh. The suspension was centrifuged at 25 x g for 3 min, and the resulting pellet was resuspended in Powell medium containing 200 µM calcium chloride to adapt cells to calcium. Thereafter, cells were centrifuged at 25 x g for 2 min, and the cell pellet was resuspended in Powell medium containing 400 µM calcium chloride solution. Test tubes were filled with 1 mM calcium chloride solution and resuspended cardiomyocytes were added. Intact myocytes were collected after centrifugation at 15 x g for 1 min. The supernatant was removed and the pellet resuspended in culture medium resulting in 40-60 % intact cells.

3.2. Culturing of cardiomyocytes

The following solutions were used for culturing isolated cardiomyocytes.

CCT Medium (sterile filtered, pH 7.4)

M199 / HEPES	x ml
Creatine	5 mM
Taurine	5 mM
Carnitine	2 mM
Cytosin-β-Arabinofuranoside	10 µM

Preincubation Medium

M199/HEPES (sterile filtered, pH 7.4)	x ml
FCS	4 % (vol/vol)
Penicillin	100 IE/ml
Streptomycin	100 µg/ml

CCT Culture medium

CCT Medium (sterile filtrated, pH 7.4)	x ml
Penicillin	100 IU/ml
Streptomycin	100 µg/ml

3.2.1. Pre-incubation of culture plates

In order to allow cardiomyocytes to attach, culture dishes were incubated at least 2 hours at 37°C with pre-incubation medium. The medium was removed before plating cells.

3.2.2. Plating of cardiomyocytes

Isolated cardiomyocytes from one heart were suspended in 24 ml of CCT culture medium, mixed homogeneously and plated at a density of approximately 5 x 10^4 cells per 35 mm culture dish. In each culture dish the portion of rod-shaped, living cells ranged from 40 to 60 %.

3.2.3. Culturing of cardiomyocytes

Plated cells were cultured for 2 hours at 37°C under CO_2-free conditions and then washed two times with CCT-culture medium to remove round and non-attached cells. This results in 90 % living, intact, rod-shaped cells. If cells should be incubated for 24 hours, gentamycin (10 µg/ml) was added to the CCT culture medium.

3.2.4. Treatment of cardiomyocytes

After washing cardiomyocytes with CCT culture medium, the cells were treated according to different protocols; e.g., phenylephrine was used to stimulate α-adrenoceptors and was applied at a concentration of 10 µM. At this concentration, no cross-reactivity to β-adrenoceptor pathways is present. Isoprenaline was used to stimulate β-adrenoceptors and was applied at a concentration of 1 µM. NFAT (500 nM) and mut-NFAT (500 nM) were added to the culture medium 5 h before stimulating the cells with phenylephrine and isoprenaline. The calcium-chelating agent BAPTA/AM (10 µM) was added to the culture medium 2 h before stimulating the cells with phenylephrine. Gö6850 was used as an inhibitor of protein kinase C at a concentration of 1 µM. Cyclosporine was used at a concentration of 1 µM. These drugs were added and cells and were incubated according to respective protocols at 37°C, 95 % humidity. For controls un-treated cells were used. Afterwards, all treated and un-treated cells were incubated at 37°C, 95 % humidity. Added substances were left in the culture dishes.

3.3. Transformation of cardiomyocytes

3.3.1. Hybridization of decoy oligonucleotides

For transformation of cardiomyocytes the following oligonucleotides were used.

Decoy oligonucleotides

NFAT decoy oligos

Forward 5´-GCCCAAAGAGGAAAATTTGTTTCATACAG-3´
Reverse 5´-CTGTATGAAACAAATTTTCCTCTTTGGGC-3´
Mut-NFAT decoy oligos
Forward 5´-GCCCAAAGATACGAATGGACTTCATACAG-3´
Reverse 5´-CTGTATGAAGTCCATTGGTATCTTTGGGC-3´

Each decoy oligonucleotide was dissolved in Tris-HCl/EDTA buffer. To hybridize complementary strands equimolar amounts of oligos (100 µM) were mixed and heated in the PCR thermocycler at 95°C. The reaction was then slowly cooled down for several minutes to room temperature. In this time complementary strands passed through the melting point and hybridized as double-stranded DNA fragments.
The double-stranded oligos contained consensus-binding sequences. These decoy oligos are capable of scavenging intracellular specific transcription factors.

3.3.2. Transformation of cardiomyocytes with decoy oligonucleotides

To increase stability of oligonucleotides against intracellular exonucleases the last 5 bases on each end were modified into phosphothioesters. For transformation of cells decoy oligonucleotides (500 nM) were added to the medium after washing of the cells with CCT culture medium. The cells were incubated 5 h at 37°C, 95 % humidity.

3.4. Determination of hypertrophic growth

3.4.1. Determination of the rate of protein synthesis

The effect of α-adrenoceptor agonist PE (10μM) on the rate of protein synthesis in ventricular cardiomyocytes from adult rat was determined by incorporation of ^{14}C-phenylalanine during 24 h.

Induction medium
CCT culture medium X ml

^{14}C-phenylalanine	0.1 μCi/ml
Penicillin	100 IE/ml
Streptomycin	100 μg/ml
Ascorbic acid	100 μM

10XPBS

NaCl	150 mM
NaH_2PO_4 X H_2O	4 mM
Na_2HPO_4 X H_2O	16 mM

The pH was adjusted to 7.4

Incorporation of phenylalanine into cells was analysed by exposing cultures to ^{14}C-phenylalanine for 24 hrs and determining the incorporation of radioactivity into the acid-insoluble cell mass. Non-radioactive phenylalanine (0.3mM) was added to the medium to minimize variations in the specific activity of the precursor pool responsible for protein synthesis.

Experiments were terminated by removal of the medium from the cultures. Cells were washed three times with 1 ml of ice-cold PBS. Subsequently, 1 ml of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) was added. Protein precipitation was performed overnight at 4°C. The next day 500 μl of TCA were taken per dish, mixed with 4 ml of scintillation liquid, and decay events per minute were counted in a β-counter. The decay events per minute of scintillation liquid without any addition were determined in a β-counter as a reference value. Radioactivity in this acid fraction presented the intracellular precursor pool. The dishes were then washed twice with 1 ml of ice-cold 10% (wt/vol) trichloroacetic acid and a third time with 1 ml of ice-cold PBS. The remaining precipitate in the culture dishes was dissolved in 1 ml of 1N NaOH/0.01% (wt/vol) SDS by incubation at 37°C overnight. For the determination of incorporated ^{14}C-phenylalanine 500 μl from cell lysate were transferred into 4 ml of scintillation liquid. Subsequently, decay events per minute were measured in a β-counter. Based on these measurements, the ratio of incorporated ^{14}C-phenylalanine into cellular protein

and the precursor pool was calculated. This rate of protein synthesis was related to total protein content determined by the Bradford method.

3.4.2. Quantification of protein by the Bradford Method

Bradford reagent

Coomassie Brillant Blue G-250	0.1 % (wt/vol)
96 % Methanol	5 % (vol/vol)
85 % Ortho-Phosphorus acid	10% (vol/vol)

To make volume 1 L with distilled water.

Total protein was measured using the method described by Bradford et al (1976). 10 µl of the cell lysate (as described in 3.4.1) was transferred into a 96-well micro-titer plate, and 200 µl Bradford reagent were added. Immediately, a change in colour, with the intensity depending on the amount of protein in each sample, was visible. The binding of the protein to the dye led to a shift in the absorption maximum from 465 nm to 595 nm. The extinction value at 595 nm was determined using a photometer. The quantification was performed by creating a standard curve using BSA which was dissolved in 1N NaOH/0.01%SDS (wt/vol) with following concentrations: 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The unknown concentration of the protein solution was then extrapolated from the standard curve using their extinction values.

3.4.3. Determination of cell size

Myocyte size was determined on micrographs digitalized by a charge-coupled-device video camera. Cardiomyocytes were stimulated for 24 h. Single cell images of these cardiomyocytes were taken. Five micrographs were taken randomly per sample, and all rod-shaped myocytes in these fields were measured. The diameter of myocytes was determined at the widest point of each myocyte, and the length of myocytes was determined at the longest point of each myocyte. Cell volumes were calculated by the following formula: Volume =

(radius) ^{2} x π x length, assuming a cylindrical cell shape. Cross-sectional area was determined by the following formula: Cross sectional area = (radius) ^{2} x π.

3.5. Determination of cell shortening

3.5.1. Sample preparation

Cell shortening of overnight pre-incubated isolated cardiomyocytes from ventricles of adult rat was measured. After 24 h incubation of cardiomyocytes (with different reagents as described) the contraction parameters were measured under application of external electrical charge.

3.5.2. Electrical stimulation of cardiomyocytes

All cell-shortening experiments were performed at room temperature. At the end of the pre-incubation period the culture-dish was placed on a microscopic stage. A special cover was used for the culture dish. This cover had four holes, which were so arranged that they formed the points of a square, whose maximum diagonal width filled up the circle of the cover. One wire that was attached to the cathode of the electro-stimulators was passed inside the cover through one of these holes so that it was immersed in the medium in the culture-dish. It was curved and bent so that it could be immersed horizontally into the medium, from where it again bent and perpendicularly extended out of the medium. The second wire, which was attached to the anode, was positioned in the same way. The two wires represented cathode and anode placed horizontally into the medium. An electrical field was developed between the two wires by passing current over them.

Biphasic electrical stimuli composed of two equal but opposite rectangular 60 V stimuli of 0.5 ms duration were applied at a frequency of 0.5, 1 and 2 Hz. Cells that did not respond to the given specific stimulation frequency were not considered in the experiments.

3.5.3. Determination of parameters of cell contraction

Cell shortening was monitored using a cell-edge detection system from “Scientific Instruments GmbH” in Heidelberg. During stimulation of the cells, culture dish was placed on the stage of a microscope. It was possible to observe the cell contraction microscopically.

Two cameras were attached to this microscope. One camera was a video camera for the observation of the cell picture on a monitor. The other one was a line camera, which was able to recognize cell borders by measuring differences in brightness as light and dark e.g. dark-light transition at the border between cell and background. For the observation of cell contraction with the line camera, the camera was positioned in such a way that both cell ends lay in the picture of the linear line. In addition to this, the culture dish was moved in such a way that the cell being examined was exactly in the center of the picture. The line camera was turned until both cell ends were covered by the line camera.

The picture from the line camera was converted into electrical signals that were presented on an oscillograph. The deflection time on the horizontal amplifier was adjusted to 0.1 ms/cm; the vertical amplifier was regulated at 5V/div. If the line camera detected changes in brightness, they were represented on the oscillograph as varying y-deflections. Those amplitudes which represented the cell borders could be identified by their horizontal movement. It was thus possible to observe the cell contraction on the oscillograph.

The oscillograph was operated as a bi-channel oscillograph. Against the second channel a strong stress or tension of the interface rested. If it was readable, the oscillograph presented a horizontal line at a certain height on the screen. If it was not readable, the oscillograph showed a horizontal line at the height of zero. The phase-contrast micrograph was recorded by video camera. The contractions of single cell were determined from consecutive frozen video frames magnifying the cell’s picture 500-fold on a video monitor screen.

The software program MUCEL provided a graph on the basis of cell lengths at different times, which showed the cell length dependent on time. This curve represented the cell contraction. The computer recognized cell shortening at the

beginning of a contraction. In each case cells were constantly paced, and five contractions were recorded every 15 s. The average of these recordings was used as one data point.

The following values were determined:

1: The maximum cell length (diastolic cell length) in micrometers.

2: The minimum cell length (systolic cell length) in micrometers.

3: The time of beginning of the contraction up to the maximum contraction "time to peak" in milliseconds.

4: The maximum contraction velocity in micrometers per second (determined from the first derivative of the contraction curve).

5: The maximum relaxation velocity in micrometers per second.

6: The time from 10% cell contraction up to the complete cell contraction in milliseconds (T10 to peak).

7: The time of the maximum contraction up to the relaxation around 90% of the cell shortening distance (R90 value).

From these parameters three further parameters were calculated:

1: The quotient ΔL/L: ΔL was the difference from diastolic to systolic cell length. Expressed in percent, ΔL/L indicates cell shortening as percent of diastolic cell length.

2: The Con_{max} represents the rate for maximum contraction velocity.

The maximum contraction speed is determined as the first temporal derivative of cell shortening and is indicated in µm/s.

3: The Rel_{max} represents the rate for the maximum relaxation velocity. It is determined as the first temporal derivative of the relaxation and is indicated in µm/s.

3.5.4. Measurement of cell contraction

Each cell was stimulated at 0.5, 1 and 2 Hz for 1 min and every 15 s cell shortening was measured. The average of these recordings was used as one data point. The measurements on individual cells were repeated four times and the median of these four measurements at a given frequency per cell was used

as the average cell shortening of individual cells. Cell lengths were measured at a rate of 500 Hz via a line camera.

3.6. Western blot

1xPBS

NaH_2PO_4 X $2H_2O$	0.004 M
Na_2HPO_4 X$2H_2O$	0.016 M
NaCl	0.150 M

Volume made 1 L by distilled water

Cell lysis buffer

Tris pH 6.7	50 mM
SDS	2 %

Prior to use the following reagents were added

Na-Vanadat, 100 mM	10 % of lysis buffer
3 Mercapto-1,2-propandiol	10 % of lysis buffer

Benzonase 1:30 diluted with distilled water

3.6.1. Lysis of cells

Cells were washed with 1 ml of ice-cold 1XPBS. 100 µl of protein lysis buffer were added in each plate. After an incubation time of 10 minutes on shaker at room temperature, 10 µl of Benzonase (1:30) was added and plates were again shaken for 10 minutes. Proteins were scraped, transferred into fresh tube and stored at -20°C.

3.6.2. SDS Polyacrylamide gel (7.5%)

Resolving gel

Distilled Water	9.55 ml
30 % Acrylamide (29:1 AA/BA)	5.25 ml

1.5 M Tris pH 8.6	4.75 ml
10 % SDS	200 µl
10 % APS	200 µl
TEMED	15µl

Stacking gel

Distilled Water	4.55 ml
30 % Acrylamide (29:1 AA/BA)	1.25 ml
0.5 M Tris pH 6.8	1.5 ml
10 % SDS	62.5 µl
10 % APS	50 µl
TEMED	5 µl

Running buffer

Glycine	192 mM
Tris	24.8 mM
SDS	3.5 mM

Distilled Water was added up to 1000 ml and pH was adjusted to 8.2-8.5

Protein loading buffer

SDS	1.0 g
DTT	0.77g
Glycerol (100%)	10 ml
Bromophenol blue	1 mg

0.5 M Tris, pH 6.8 was added to make the volume 20 ml.

Preparation of samples

Protein sample	10 µl
Protein-loading buffer	10 µl

Samples were incubated for 5 minutes at 95°C and then loaded on gel.

3.6.2.1. Preparation of gel

The above-mentioned resolving and stacking gel reagents were for one 7x10 cm gel, 0.75-1.00 mm thick. Glass plates were assembled by placing a spacer at both sides and sealed at the bottom. APS and TEMED were added just prior to pouring the gel, as these reagents promote and catalyse the polymerisation of acrylamide. The resolving gel mix was poured between the assembled glass plates leaving sufficient space for the stacking gel to be added later. The gel was gently overlaid with distilled water and the gel was allowed to polymerise for 20 minutes. After polymerisation, distilled water overlay was removed, the remaining space was filled with stacking gel and comb was inserted immediately. After the stacking gel was polymerised, the comb was removed and the wells were rinsed with running buffer to remove the un-polymerised acrylamide. At least 1 cm of stacking gel was present between the bottom of the loading wells and the resolving gel.

The samples were prepared and loaded. In one lane 20 μl of rainbow marker was loaded. The gel was run at 200 volts for 2-3 hours until the bromophenol blue reaches the bottom of the resolving gel. Then the gel was used for western blotting.

3.6.3. Blotting of proteins

Western blot buffer A (Cathode buffer)

25 mM Tris	3.03 g
40 mM 6-amino-hexanoic acid	5.25 g

Distilled water was added to a total volume of 800 ml and pH was adjusted to 9.4, then

20 % Methanol	200 ml

Western blot buffer B (Anode buffer)

30 mM Tris	3.63 g

Distilled water was added to a total volume of 1000 ml and pH was adjusted to 10.4, then

20 % Methanol	200 ml

Western blot buffer C (10 X Anode buffer)

300 mM Tris	36.3 g

Distilled water was added to a total volume of 1000 ml and pH was adjusted to 10.4, then

20 % Methanol	200 ml

3.6.3.1. Preparation of blotting chamber and transfer of protein to membrane

9 pieces of whatman papers were cut to the size of gel. 3 papers were soaked with buffer C and placed at the bottom of chamber. Above 3 papers soaked with buffer B were placed. PVDF membrane of equal size as the gel was soaked in buffer B and placed above. Upon completion of electrophoresis, the gel was removed from glass plates. The gel was placed above the membrane and 3 papers soaked with buffer A were placed at the top. Blot was run at 160 mA per two gels for 2 h. Gel was removed, membrane was dried between two sheets of paper and stored at 4°C or used immediately.

3.6.4. Incubation of membrane with antibodies

Dried membrane was soaked in 100% methanol; membrane was washed 2 times with washing buffer and twice with1xPBS. Each washing was for 10 minutes

Blocking buffer

PBS buffer	1x
Tween20	0.05%
BSA	3%

Washing buffer

PBS	1X
Tween20	0.05%

3.6.5. Antibody dilutions

SERCA2 (C-20) antibody dilution

SERCA2 antibody	1:1000
PBS	1X
Tween20	0.05%
BSA	3%

Bovine anti-goat IgG-AP dilution

Bovine anti-goat-AP-conjugated	1:1000
PBS	1X
Tween20	0.05%
BSA	3%

Dilution of anti-Actin antibody

Anti-Actin antibody	1:2000
PBS	1X
Tween20	0.05%
BSA	3%

Dilution of anti rabbit IgG-AP conjugated antibody

Anti rabbit antibody	1:2000
PBS	1X
Tween20	0.05%
BSA	3%

Membrane was incubated in blocking buffer at room temperature for 1 hour, washed 2 times with washing buffer and two times with 1XPBS. Membrane was

incubated with first antibody either SERCA2 or actin for 2 h at room temperature or over night at 4°C on shaker. Membrane was washed 2 times with washing buffer and twice with 1xPBS. Membranes for SERCA detection were incubated with bovine anti-goat antibody and membranes for actin with anti-rabbit IgG-AP conjugated antibody for 1-2 hours on shaker at room temperature. Membrane was washed 2 times with washing buffer and two times with 1XPBS, 10 minutes for each washing step.

Substrate buffer

Tris	100 mM
$MgCl_2X6H_2O$	5 mM
NaCl	10 mM
pH 9,55	

3.6.6. Staining solution (light sensitive)

Substrate buffer	40ml
BCIP	10.0mg
NBT	13.2mg

The membrane was incubated in staining solution in dark place until protein bands were visible. Staining was stopped with tap water and the membrane was dried. Photograph of the membrane was taken by imaging device (BioRad) and bands were quantified by using Quantity One program.

3.7. RNA isolation

3.7.1. Harvesting of cells

Cultured cells (35mm cell culture dishes) were scraped with a disposable cell scraper and collected in tubes. The cell solutions were centrifuged at 3000 rpm for 3 minutes at room temperature. The pellets were dissolved in 1 ml of ice-chilled 1xPBS and transferred into fresh tubes. The cells were centrifuged at

2500 rpm for 3 minutes at room temperature and cell pellets were stored at -20°C.

3.7.2. RNA isolation

Total RNA was extracted from cells using TRIzol® reagent. Each sample was dissolved in 1.0 ml of TRIzol® reagent by pipetting up and down, and then mixed. Following 5 min incubation at room temperature, 200 µl of chloroform were added to these samples. Samples were shaken vigorously by hand for 15 sec and incubated at room temperature for 2-3 min and centrifuged at 12000 rpm for 15 min at 4°C. The RNA containing upper phase was collected in a new tube. The RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropanol. RNA was incubated for 10 min at room temperature and centrifuged at 12000 rpm for 15 min at 4°C. The RNA pellet was washed with 1 ml of 70% ethanol. The samples were centrifuged at 12000 rpm for 15 min at 4°C. The RNA pellet was dried and dissolved in DEPC treated water.

3.7.3. DNAse treatment of RNA

DNAse buffer

HEPES	80mM
NaCl	10mM

Volume made up by DEPC water.

DNAse reaction mix

10XDNase buffer	5µl
$MgCl_2$, 50mM	5µl
DTT, 0.1M	5µl
RNAse inhibitor	1µl
DNAse, RNAse free	1µl
Final volume	17µl

The RNA pellet was dissolved in 33 µl DEPC treated water and 17 µl of DNAse reaction mix was added. Samples were incubated for 1 h at 37°C. These samples were placed at -80°C overnight or used directly. 50 µl of DEPC treated water was added in each sample for making final volume of each sample 100 µl. 100 µl of phenol chloroform solution (1:1) was added in each sample. Samples were mixed and centrifuged at 13000 rpm for 10 minutes at 4°C. The RNA containing upper phase was collected in fresh eppendorf tubes. The RNA was precipitated by adding 10 µl of NaAc (3M) and 250 µl of absolute ethanol. The samples were shaken gently by hand and centrifuged at 13000 rpm for 30 minutes at 4°C. The RNA pellet was washed with 500 µl of 70% ethanol, mixed and centrifuged at 13000 rpm for 15 min at 4°C. The RNA pellet was dried and re-dissolved in 20 µl of DEPC treated water and stored at -80°C.

RNA concentration and purity were determined photometrically. For this analysis 3 µl of probe were mixed in 1 ml of distilled water and absorbance at 260 nm and 280 nm was measured. For the estimation of purity, A_{260}/A_{280} ratio was calculated. The ratio around 1.8 is considered to be good quality RNA in our experiment.

3.8. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to determine expression of messenger RNA (mRNA), total RNA was converted into complementary DNA (cDNA) by reverse transcriptase. cDNA was then amplified by PCR, and analysed by agarose gel electrophoresis.

3.8.1. cDNA synthesis

Complementary DNA was synthesized from total RNA using reverse transcriptase (MMLV-RT). One µg of total RNA was combined with nuclease-free water for a final volume of 5 µl per reaction. This mixture was denatured at 60°C for 10 min, followed by rapid cooling. After a short spin, 5 µl of the reverse transcription reaction mix was added and incubated at 37°C for 1 h, followed by inactivation of enzyme at 95°C for 10 minutes. After synthesis, cDNA samples were either used immediately for PCR, or stored at -20˚C.

Reverse transcriptase reaction mix

5XRT-buffer	2 µl
Oligo dt	1 µl
dNTP's, 10 mM	1 µl
DTT, 0.1 M	0.5 µl
RNAsin	0.2 µl
MMLV-RT	0.3 µl
Final volume	5 µl

3.8.2. Polymerase Chain Reaction (PCR)

To analyse mRNA expression in cardiomyocytes, PCR was performed on cDNA samples by use of Taq polymerase. cDNA was amplified using mRNA-specific primers (as described below). Primers were designed from sequence data available in the GenBank. The primer sequence was checked using the NCBI BLAST search for probable similarity with unrelated genes. For PCR, 20-22 bp long primers were designed, AT and GC content was checked and the difference in the melting temperature (Tm) between the forward and reverse primers was kept not more then 2-4ºC. Each primer pair was tested with several annealing temperatures depending on the Tm of the primer pair to get a single and specific PCR band.

PCR primers

Beta-Actin forward	5´ GGCTCCTAGCACCATGAAGA 3´
Beta-Actin reverse	5´ ACTCCTGCTTGCTGATCCAC 3´
HPRT forward	5´ CCAGCGTCGTGATTAGTGAT 3´
HPRT reverse	5´ CAAGTCTTTCAGTCCTGTCC 3´
SERCA forward	5´ CGAGTTGAACCTTCCCACAA 3´
SERCA reverse	5´ AGGAGATGAGGTAGCCGATGAA 3´

PCR reaction was done in 0.2 ml thin wall tubes in thermocycler. Negative control without template was performed to check for self-annealing of primer pairs and for genomic DNA contamination.

The polymerase chain reaction (PCR) allows amplification of DNA fragments due to repetitive cycles of DNA synthesis. The reaction uses four dNTP's, heat-stable DNA polymerase (Taq) and two specific primers, which hybridize to sense and antisense strand of the template DNA fragment. Each cycle consists of three reactions that take place under different temperatures. First the double stranded DNA is converted into its two single strands (denaturation at 93ºC). They function as templates for the synthesis of new DNA. Second, the reaction is cooled (57-59ºC) to allow the annealing of primers to the complementary DNA strands (hybridization). Third, DNA polymerase extends both DNA strands at 72ºC starting from the primer sequence (DNA synthesis).

PCR mix

Nuclease-free water	6.3 µl
10XPCR-buffer	1.0 µl
β-actin forward, 100 µM	0.15 µl
β-actin reverse, 100 µM	0.15 µl
dNTP's, 10mM	0.4 µl
$MgCl_2$, 50mM	0.3 µl
Taq DNA polymerase	0.2 µl
Volume of cDNA added	1.5 µl
Total PCR reaction volume	10 µl

The tubes were flicked to mix and microfuged briefly before placing samples in the thermocycler.

The thermocycler's program for β-actin primers was as follows:

Activation of HotstarTaq	93ºC	5 min
Denaturation	93ºC	30 s

Annealing	58ºC	30 s
Extension	72ºC	45 s
Final extension	72ºC	10 min
Cycles	45	

After the amplification, PCR products (10 µl) were electrophoretically analysed in a 2% agarose gel.

3.8.3. Agarose gel electrophoresis

50XTAE buffer (1L)

2 M Tris base	242g
1 M Glacial acetic acid	57.1ml
0.5 M EDTA, pH 8	100 ml

The final pH 7.2 was adjusted.

TE

1 M Tris pH 8	10 mM
0.5 M EDTA	1 mM

10 X DNA loading buffer dye

Glycerol	30 %
BPB	0.25 %
Xylene cyanol	0.25 %

Rest of the volume made up by TE.

PCR products were analysed on 2% agarose gel in 1XTAE buffer, containing 0.5 µg/ml ethidium bromide. The DNA samples were mixed with loading buffer. Gel was run in 1XTAE buffer for 45-60 min at 80 V. The size of DNA fragments was determined by loading of 100 bp DNA ladder marker prior to electrophoresis. DNA bands were visualized under UV-Transilluminator; mRNA that produced specific DNA bands was used in real time RT-PCR.

3.9. Real-time RT-PCR

For semi-quantitative estimation of mRNA expression real-time RT-PCR analysis was performed. 3 µl of 1/10 volume of cDNA was mixed with 17 µl of reaction volume containing SYBR Green PCR mix and sequence-specific oligonucleotide primers.

Real time PCR mix

ABsolute SYBR Green Fluorescein Mix	10 µl
Nuclease-free water	6.4 µl
Forward primer, 100 µM	0.3 µl
Reverse primer, 100 µM	0.3 µl
Volume of 1/10 cDNA added	3.0 µl
Total real-time PCR volume	20 µl

The thermocycler's program for HPRT primers was as follows:

Activation of Taq	95ºC	15 min
Denaturation	95ºC	30 s
Annealing	63ºC	30 s
Extension	72ºC	30 s
Cycles	45	

The thermocycler's program for SERCA primers was as follows:

Activation of Taq	95ºC	15 min
Denaturation	95ºC	30 s
Annealing	57ºC	30 s
Extension	72ºC	30 s
Cycles	45	

All real-time reactions were done in icycler (Bio-Rad), and analysis was performed with the accompanying software. At the end of the PCR cycle, a dissociation curve was generated to ensure the amplification of a single product. The threshold cycle time (Ct values) for each gene was determined. Relative mRNA levels were calculated based on the Ct values and normalized to house keeping gene HPRT.

The icycler contains a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR reaction. As DNA is synthesized, more SYBR Green will bind and the fluorescence will increase.

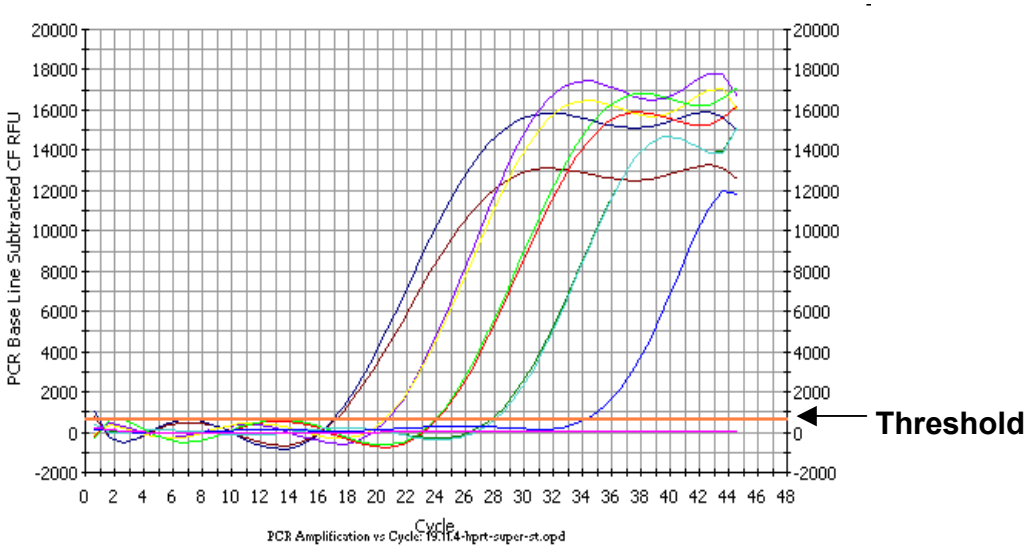


Fig.1: Amplification curves of real time PCR

Figure: 1 shows a series of 10-fold dilution of a sample of cDNA. As the sample is diluted, it takes more cycles before the amplification is detectable. In real time PCR, cycle number at which the increase in fluorescence (and therefore cDNA) is logarithmic is determined. This is shown by the horizontal line in figure 1 called threshold. In this study the same threshold for all samples of the same experiment on the same plate was used. The point at which the fluorescence

crosses the threshold is called the Ct. More diluted samples result in greater Ct values.

From such series of 10-fold dilutions of a cDNA sample a standard curve was calculated to check the efficiency and specificity of primers and other reagents (Fig 2). Standard curves for the housekeeping gene (reference gene HPRT) and also one for the gene of interest whose expression we thought may change under the experimental conditions (target gene SERCA) were done. We normally just did a single point for each dilution of the standard curve, since that gave a series of points, which fit very well to a straight line.

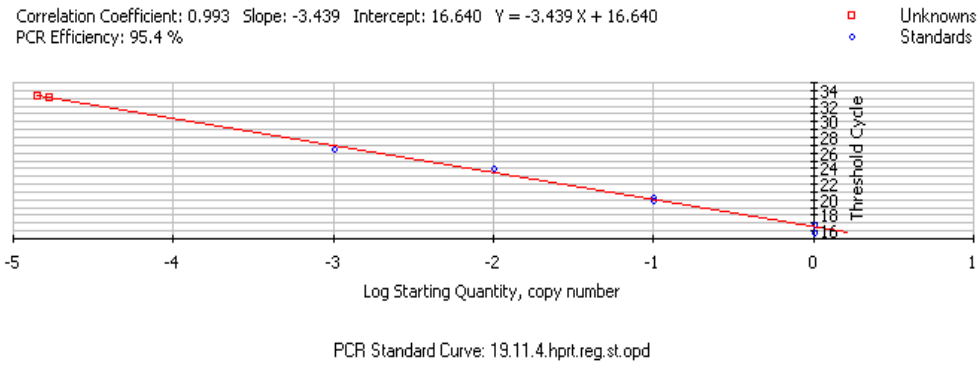


Fig.2: PCR standard curve

The excellent fit of the standard curve data to a straight line and a perfect fit should have efficiency near to 100 %, slope around -3.5 and correlation coefficient around 1.000.

In real-time PCR using SYBR green binding to amplify cDNA, we are simply measuring the fluorescence increase as the dye binds to the increasing amount of DNA in the reaction tube. To check if the correct PCR product is synthesized in real time PCR at the end of the run melting curves were determined.

Therefore, at the end of the amplification reactions, the temperature is raised continuously, and changes in fluorescence are measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The melting temperature of a DNA double helix depends on its base composition

and its length. All PCR products for a particular primer pair should have the same melting temperature, unless there is contamination, mispriming, primer-dimer artifacts, or some other problem.

The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm) of the fragments. In Fig.3 melting curves are shown.

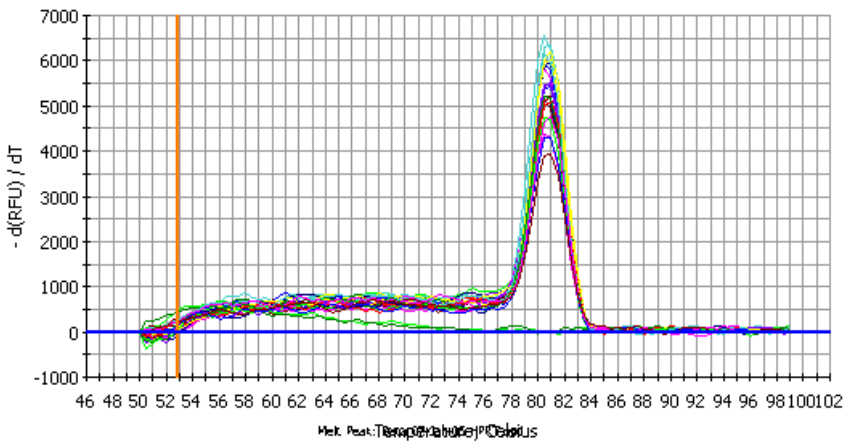


Fig.3: Melting curve

In this melting curve, all samples were run with the same primer pair. There are no signs of primer-dimer artifacts in samples.

3.10. Retardation assay

Retardation assay is a technique to determine protein-DNA interaction. The interaction of proteins with DNA is central for the control of many cellular processes including transcription. Retardation assay technique is based on the observation that protein-DNA complexes migrate slower than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis. Therefore, the rate of DNA migration is shifted or retarded upon protein binding. The DNA is labeled with fluorescent dye and can be detected by scanning in a fluo-imager.

3.10.1. Fluorescence labeling of oligonucleotides

The following double-stranded NFAT oligonucleotide was labelled with fluorescent dye:

NFAT 5´-GCCCAAAGAGGAAAATTTGTTTCATACAG-3´

This sequences bind specifically to the transcription factor NFAT in the retardation assay.

Fluorescence labelling mix

NEB buffer No. 4 (10x)	5 µl
$CoCl_2$ (2.5 mM)	5 µl
20ng Oligo(NFAT)	4 µl
Cy3-dCTP (10mM)	2.5 µl
Terminal Transferase (20 U/µl)	0.5 µl

Deionised H_2O was added to make final volume 50µl

The oligonucleotides were purchased commercially.

Procedure of fluorescence labelling of oligonucleotides

All labeling reactions were performed in brown tubes to protect the dye against light. The reaction tube was incubated for 15 minutes at 37°C. The reaction was stopped by addition of 10 µl of 0.2 mM EDTA pH 8.0 and stored at -20°C.

3.10.2. Nuclear extraction

Nuclear extraction buffers

For the nuclear extraction following buffers were used:

1 X Swelling buffer

Tris-hydroxymethylaminomethane (Tris)/HCl (pH 7.9)	10 mM
KCl	10 mM
$MgCl_2$	1 mM
Dithiothreitol	1 mM

1 X Homogenization buffer

Sucrose	300 mM
Tris (pH 7.9)	10 mM
$MgCl_2$	1.5 mM
DTT	1 mM
Triton X-100	0.3 % (vol/vol)

10 x storage buffer

NaCl	300 mM
KCl	50 mM
HEPES (pH 7.5)	10 mM
EDTA	1 mM
Dithiothreitol (DTT)	1 mM
Phenyl methyl sulfonyl fluorid (PMSF)	1 mM
Glycerol	20 % (vol/vol)

Cardiomyocytes with different treatments were harvested with 1x PBS as described above (3.7.1). For generation of nuclear extracts 2 dishes of cells were harvested and cells dissolved in 400 µl of swelling buffer. After incubation for 30 min on ice, nuclei were pelleted by centrifugation at 900 rpm for 10 min at 4°C. The upper phase was removed and pellets were suspended in 400 µl homogenization buffer for 5 min on ice, homogenized with 8 strokes in a glass homogenizer, and again centrifuged at 900 rpm for 10 min at 4°C. To the pellet 40 µl of 1 x storage buffer was added and incubated on ice, every 5 min vortexes. After 30 min extracts were centrifuged at 13000 rpm for 5 min at 4°C. The resulting supernatants contained the nuclear extract and were stored at -80°C.

3.10.3. Binding reaction of oligonucleotides with specific proteins

Binding Buffer

Glycerin	10 % (vol/vol)
Tris/HCl	20 mM
KCl	5 mM
$MgCl_2$	5 mM
DTT	3 mM
PMSF	0.2 mM

10 µl of nuclear extracts were mixed with 4.5 µl binding buffer and 0.5 µl of non-specific DNA (poly (dIdC); 1 mg/ml), and incubated at 30°C for 15 minutes. Thus non-specific DNA binding proteins could be intercepted. 1 µl of fluorescence labelled oligonucleotides together with 1.5 µl of binding buffer were added to the above reaction tubes and incubated at 30°C for 30 min. During this time specific proteins will bind to their consensus binding sites of the oligos.

3.10.4. Retardation assay gel electrophoresis

For gel electrophoresis the following solutions were used.

Solutions for retardation gel electrophoresis

Distilled water	35 ml
Acrylamide/Bisacrylamide (80%, 79:1)	4 ml
Glycerine	1 ml
100 x RA-buffer	400 µl
Ammoniumpersulphate 10 % (wt/vol)	200 µl
TEMED	34 µl

100x RA-buffer, pH 7.9

Tris/HCl	670 mM
Sodium accetate	330 mM
EDTA	100 mM

TE

1 M Tris pH 8	10 mM
0.5 M EDTA	1 mM

10 X marker dye

Glycerol	30 %
BPB	0.25 %
Xylen cyanol	0.25 %

Rest of the volume made up by TE.

The reaction mix was loaded on 4% denaturing polyacrylamide gels. For preparation of these gels two glass plates were cleaned with detergent and degreased with alcohol (100 %). Between the plates now two spacers were placed. The plates were clamped in a gel-casting device and sealed at the bottom. Subsequently, freshly prepared retardation gel solution was poured bubble free into the area between the two plates. Then the comb was set. The gel polymerized in approximately 30 min at room temperature.

The comb was removed; the glass plates were transferred from the gel-casting device into the electrophoresis chamber. The chamber was filled in such a way with 1xRA-buffer that the gel had both above and down a bubble free contact to the buffer. Now the labeled reaction samples and distance indicator bromophenol blue were loaded, and the electrophoresis chamber was attached to the power supply unit. Electrophoresis was performed at 4°C and 150 V for 2 h. Gels were scanned in fluo-imager and images were evaluated.

4.Results

4.1. Analysis of NFAT involvement in hypertrophic growth of ventricular cardiomyocytes of rat under stimulation with α-adrenoceptor agonist PE.

The transcription factor AP-1 induces hypertrophy in cardiomyocytes. Since NFAT can co-operate with AP-1 it was tested if NFAT is induced and involved in hypertrophic growth conditions.

4.1.1. NFAT is activated under PE

Activation of NFAT binding under PE was determined by retardation gel assays. Cardiomyocytes of adult rat were stimulated with PE (10 μM) for 2 h. Nuclear extracts of cardiomyocytes were isolated and retardation assay was performed to determine the induction of NFAT binding activity. Therefore, nuclear extracts were incubated with fluorescence labeled NFAT oligonucleotides, which contain NFAT binding sequences. When binding active NFAT protein is present in the nuclear extracts it will bind to these oligonucleotides. When run on retardation assay gels these binding results in band shifts. Nuclear extracts of PE induced cells showed a strong band shift as compared to the control (Fig.4.1.1.A). NFAT binding was increased by PE to 136 ± 8.2 % compared to controls (Fig.4.1.1.B). This demonstrates that PE induces NFAT binding activity in ventricular cardiomyocytes of rat.

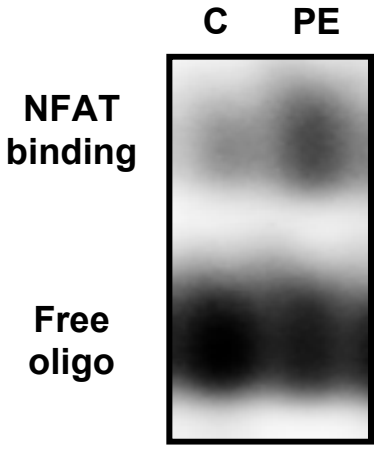


Fig: 4.1.1.A. NFAT activation by phenylephrine. This figure represents a retardation gel assay that indicates enhanced NFAT binding activity in cells treated with phenylephrine (PE, 10 µM) as compared to untreated controls (C).

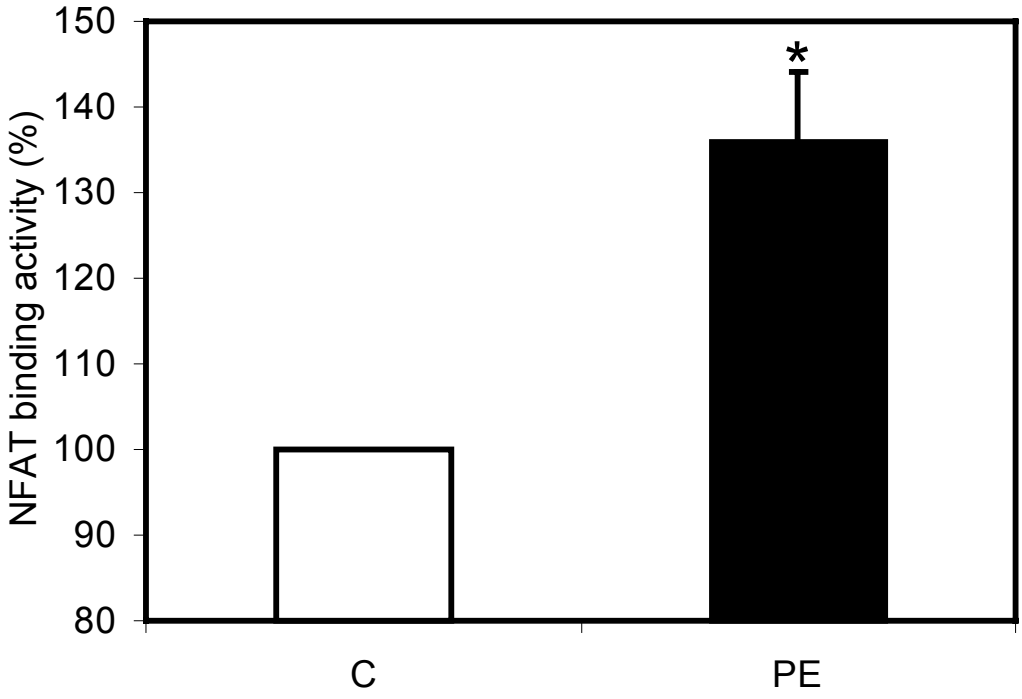


Fig: 4.1.1.B. NFAT-activation by phenylephrine. Quantitative analysis of retardation gels indicating enhanced NFAT binding activity in cells treated with phenylephrine (PE, 10 µM). Data are means ± S.E.M. of n = 5 experiments, * p <0.05 vs. control (C).

4.1.2. NFAT is inhibited by decoy oligonucleotides

For intracellular scavenging of NFAT binding cardiomyocytes were transformed with 500 nM of NFAT decoy oligonucleotides (containing the specific binding sequences for NFAT) or Mut-NFAT decoy oligonucleotides (void of specific binding site). Then cells were stimulated with PE (10 µM) in the presence or absence of NFAT and Mut NFAT decoy oligonucleotides. After 2 h nuclear

extract of cardiomyocytes were isolated. The nuclear extracts were incubated with fluorescence labeled NFAT oligonucleotides, which have NFAT binding sequences. The samples were run on retardation assay gels. These experiments showed that NFAT was activated in cells treated with PE (136 ± 8.2 %). This enhanced NFAT activity was reduced by NFAT decoy oligos (92 ± 6.5 %) but not by Mut-NFAT decoy oligos (126 ± 7 %) as compared to controls (Fig.4.1.2.B).

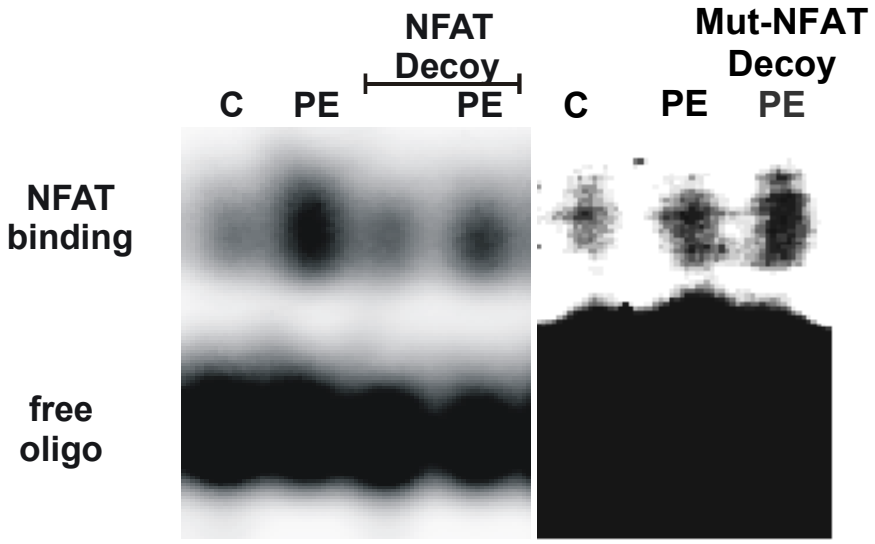


Fig: 4.1.2.A. Inhibition of NFAT by NFAT decoy oligonucleotides. Retardation gels indicating NFAT binding activity under control conditions (C), in cells treated with phenylephrine (PE, 10 μM), cells transfected with oligo decoys directed against NFAT (NFAT decoy), cells treated with mutated NFAT oligo decoys (Mut-NFAT decoy), or combination of these supplements.

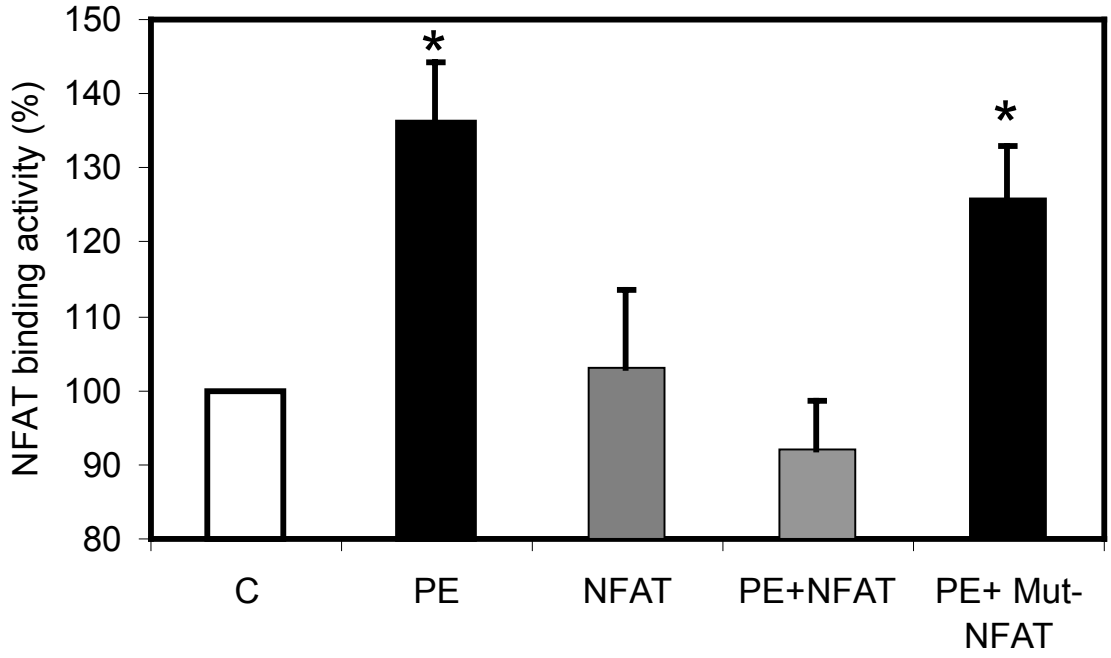


Fig: 4.1.2.B. Quantitative analysis of retardation gels showing NFAT inhibition by NFAT decoy oligonucleotides. Data are means ± S.E.M. of n = 5 experiment, * p <0.05 vs. control (C).

4.1.3. NFAT is not involved in hypertrophic growth induced by PE

Since α-adrenoceptor agonist phenylephrine activates NFAT, we assumed that NFAT might be involved in PE-induced hypertrophic growth. To test this hypothesis cardiomyocytes were transformed with 500 nM of NFAT decoy oligonucleotides that have been shown to intracellular inhibit NFAT binding activity, or with Mut-NFAT decoy oligos. Cells were then stimulated with PE (10 μM) overnight. This NFAT inhibition did not reduce the hypertrophic growth effect of PE (Fig.4.1.3.A). Still PE increased the rate of protein synthesis, determined by incorporation of radioactive labeled ^{14}C-phenylalanine (0.1 μCi/ml) during 24 h. The rate of protein synthesis was 133 ± 2.7 % under PE, 115 ± 7 % under NFAT decoy 145 ± 5.4 % under PE in presence of NFAT decoy and 149 ± 5.3 % under PE in presence of Mut-NFAT decoy oligo as compared to 100 % in controls (Fig.4.1.3.A). This rate of protein synthesis was related to total protein contents determined by Bradford method. In the presence of Mut-NFAT decoy

oligo hypertrophic growth was also not inhibited. These results demonstrate that NFAT, although it is induced by PE, it is not a mediator of PE-induced hypertrophy.

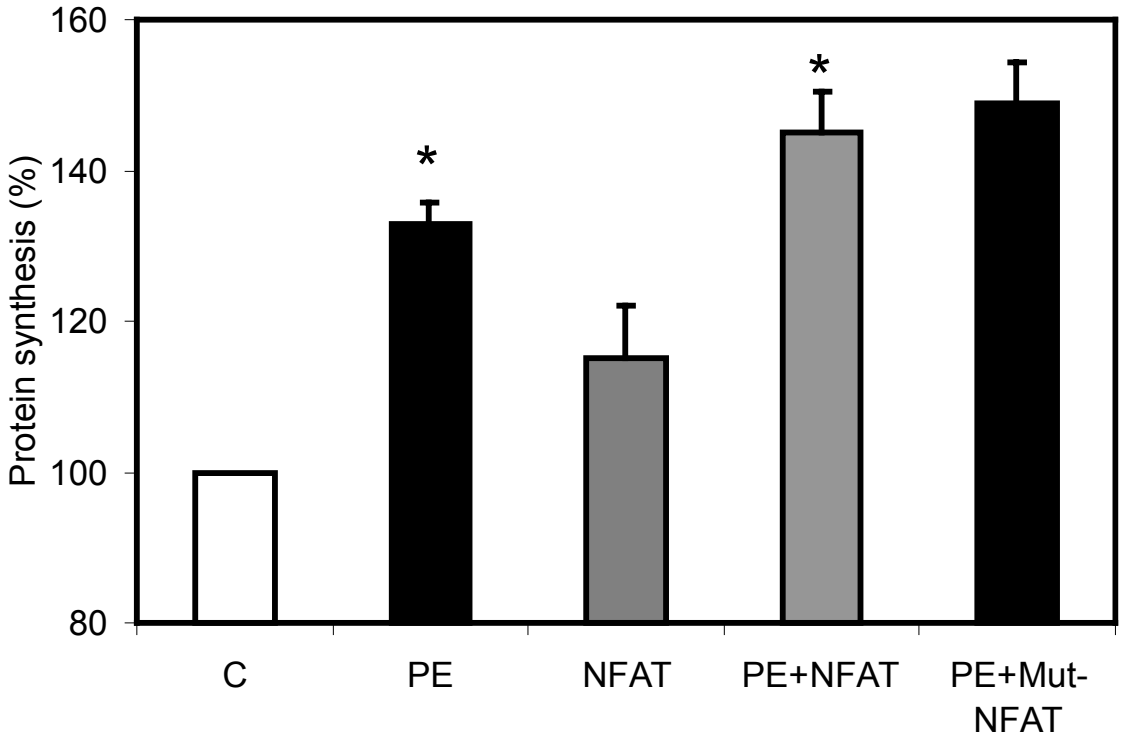


Fig: 4.1.3.A. Effect of NFAT activation on phenylephrine-induced hypertrophic growth response. Impact of PE (10 µM) in cardiomyocytes transformed with NFAT and Mut-NFAT oligo decoys was determined by ^{14}C-phenylalanine incorporation during 24 h. Data are means ± S.E.M. of n = 4 experiments, * p <0.05 vs. control (C).

As another parameter of hypertrophic growth, cell size was determined. Again cardiomyocytes were transformed with NFAT and Mut-NFAT decoy oligonucleotides for 5h. Then cardiomyocytes were exposed to PE for 24 h. One

plate of NFAT decoy oligos treated cells and one plate of Mut-NFAT decoy oligos transformed cells were kept without PE exposure. Myocyte size was determined on micrographs digitalized by a charge-coupled device camera. Diameters of myocytes were determined. Cross-sectional area and cell volume was calculated. The cross-sectional area of cells treated with PE in presence of NFAT decoy oligos was increased to 312.12 ± 5.64 % as compared to 267.51 ± 5.06 % in myocytes only treated with NFAT decoy oligos. The cross-sectional area of cells treated with PE and Mut-NFAT decoy oligos also increased to 337.67 ± 8.06 % compared to 241.23 ± 5.64 % in cells transformed with Mut-NFAT decoy oligos in absence of PE stimulation (Fig 4.1.3.C). The cell volume was increased to 29482.9 ± 1083.9 % by PE in presence of NFAT as compared to 25161.6 ± 1052.3 % in cells treated with NFAT decoy oligos only. In presence of Mut-NFAT decoy oligos PE increased the cell volume to 30451.1 ± 1459.5 % as compared to 22885.5 ± 1152.5 % in Mut-NFAT decoy transformed cells (Fig.4.1.3.D). These results show that cross-sectional area and cell volume is increased by PE (Fig.4.1.3.B & D). This parameter of hypertrophy is not blocked by NFAT decoy oligonucleotides.

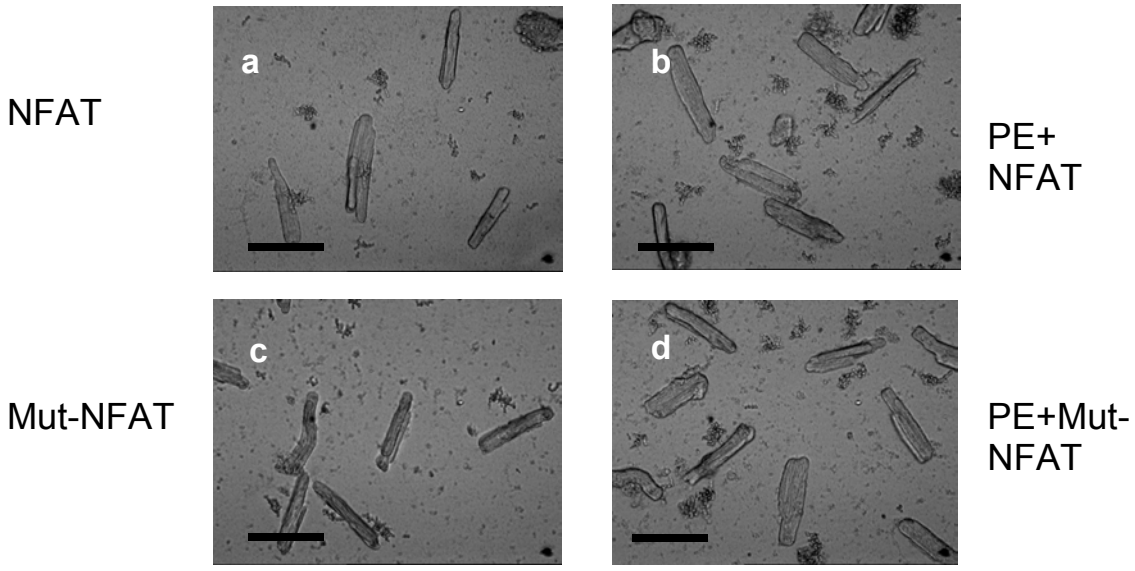


Fig: 4.1.3.B. Impact of phenylephrine on cell size in cardiomyocytes transformed with decoy oligos directed against NFAT or Mutant NFAT decoy oligos. a-d are representative pictures of cardiomyocytes transfected with NFAT (a+b) or Mutant NFAT decoy oligos (c+d). Cells were either kept without phenylephrine (PE, 10 µM) (a+c) or exposed to phenylephrine (b+d) for 24 h. The bars represent 100 µm.

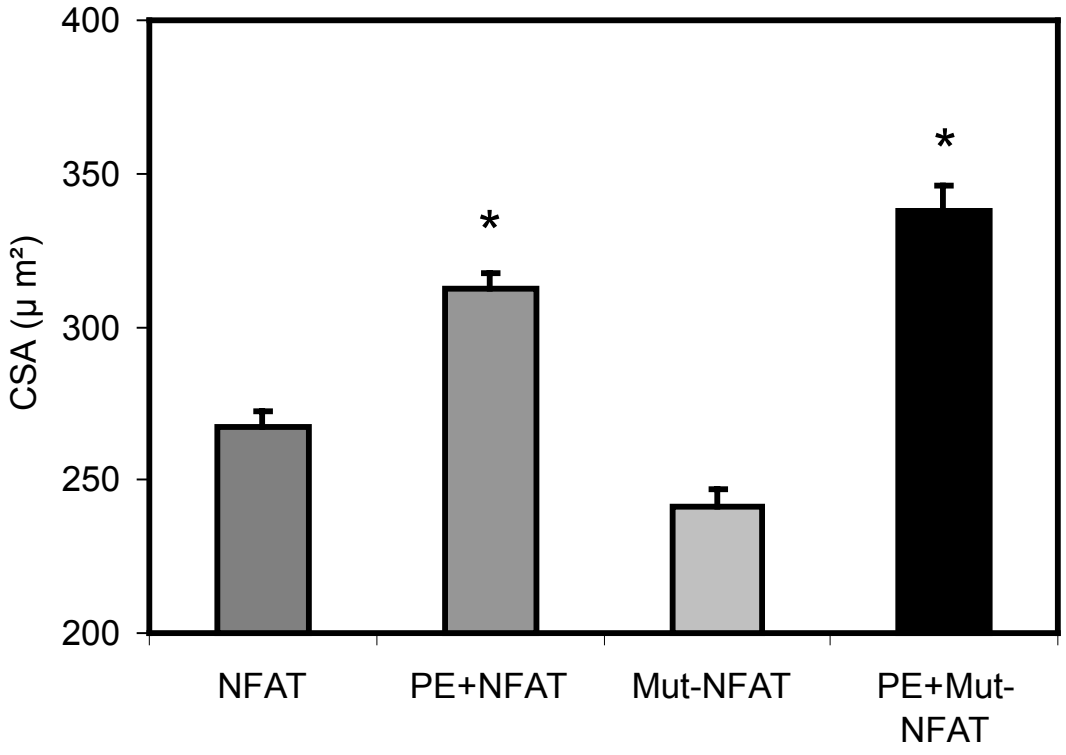


Fig: 4.1.3.C. Quantitative analysis of cross sectional areas of the cells. Cells were treated as explained under Fig.4.1.3.B. Then cross sectional area was determined.

Data are means ± S.E.M. from n=120 cells, * $p<0.05$ vs. cells not treated with PE.

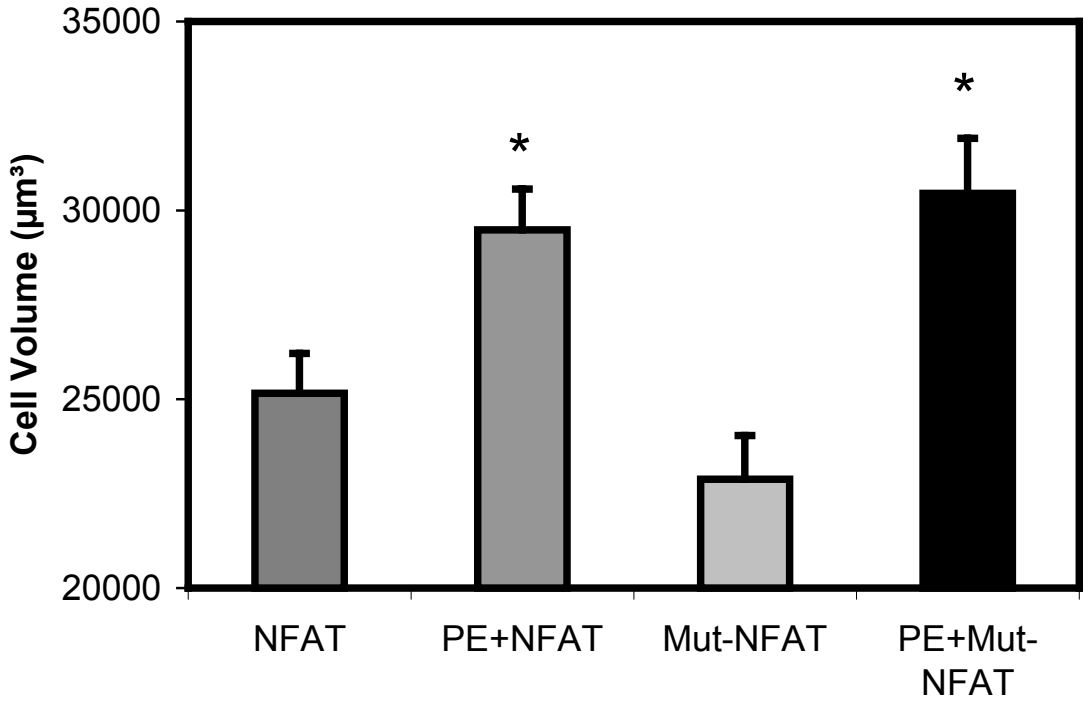


Fig: 4.1.3.D. Quantitative analysis of cell volumes.

Data are means ± S.E.M. from n=120 cells, * $p<0.05$ vs. cells not treated with PE.

4.2. Effect of PE on cell contraction

Besides its hypertrophic effects PE may also influence cell contraction. To analyse this, contraction of cardiomyocytes due to electrical stimulation was analysed after 24 h exposure to PE.

4.2.1. Cell shortening is reduced at low beating frequency (0.5 Hz)

Cardiomyocytes were incubated for 24 h in the presence of PE, then the drug was washed out and cell shortening was analysed by using cell-edge detection system. Cells were stimulated at 0.5, 1 or 2 Hz for 1 minute, and every 15 s cell shortening was measured. 24 h exposure of cardiomyocytes to PE reduced cell shortening at low beating frequency at 0.5 Hz to 4.82 ± 0.62 % as compared to

7.25 ± 0.4 % in controls. However, when beating frequency was increased reduced cell shortening was less pronounced and no more significant at 2 Hz (6.99 ± 1.32 %) as compared to 8.48 ± 1.35 % in controls (Fig.4.2.1.).

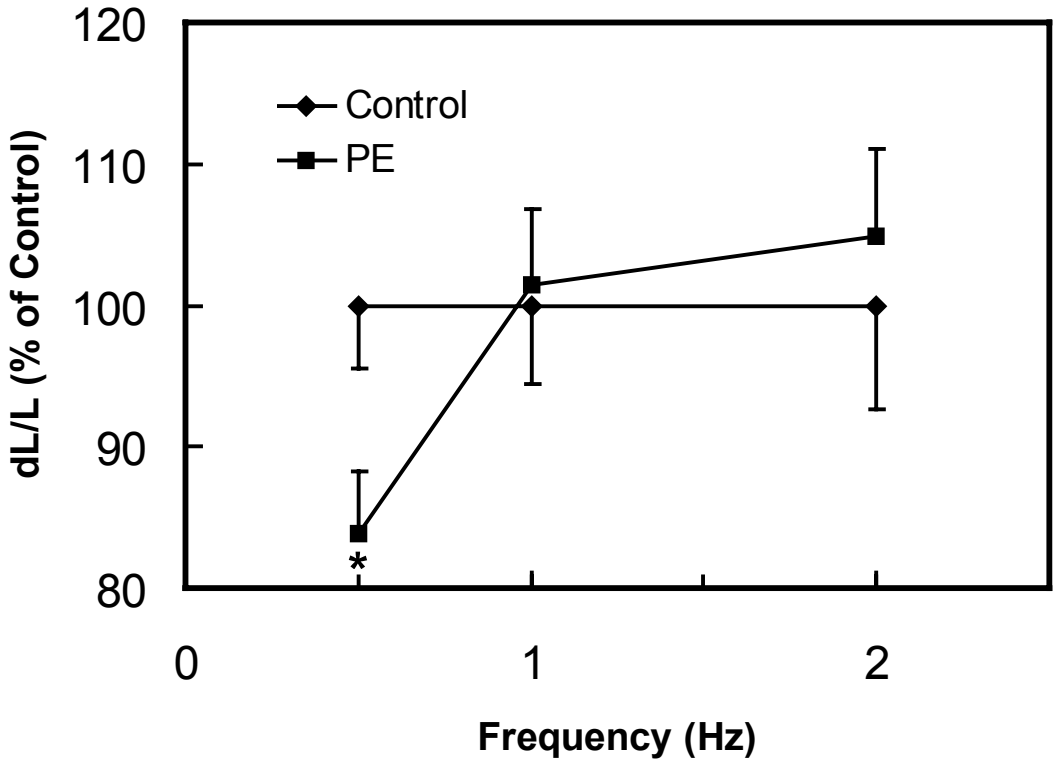


Fig: 4.2.1. The impact of α-adrenoceptor stimulation on cell shortening at various beating frequencies. Cells were kept over night under control conditions (control) or in presence of phenylephrine (PE, 10 μM). Data are expressed as percent cell shortening relative to the diastolic cell lengths. Data are means ± S.E.M. (n=24 cells, 4 experiments), * $p<0.05$ vs. control.

4.2.2. Relaxation velocity under PE is enhanced at high beating frequency at 2.0 Hz

Cardiomyocytes were pre-incubated with PE (10 μM) for 24 h. Relaxation velocity of these cells was enhanced with increasing frequencies of electrical stimulation. The relaxation velocity of PE stimulated cells at 0.5 Hz was 246 ± 122 % as compared to 145 ± 22 % in controls, and increased to 897 ± 296 % at 2 Hz compared to 286 ± 45 % in controls. Therefore, the relaxation velocity at high beating frequencies is enhanced in PE treated cells (Fig.4.2.2.).

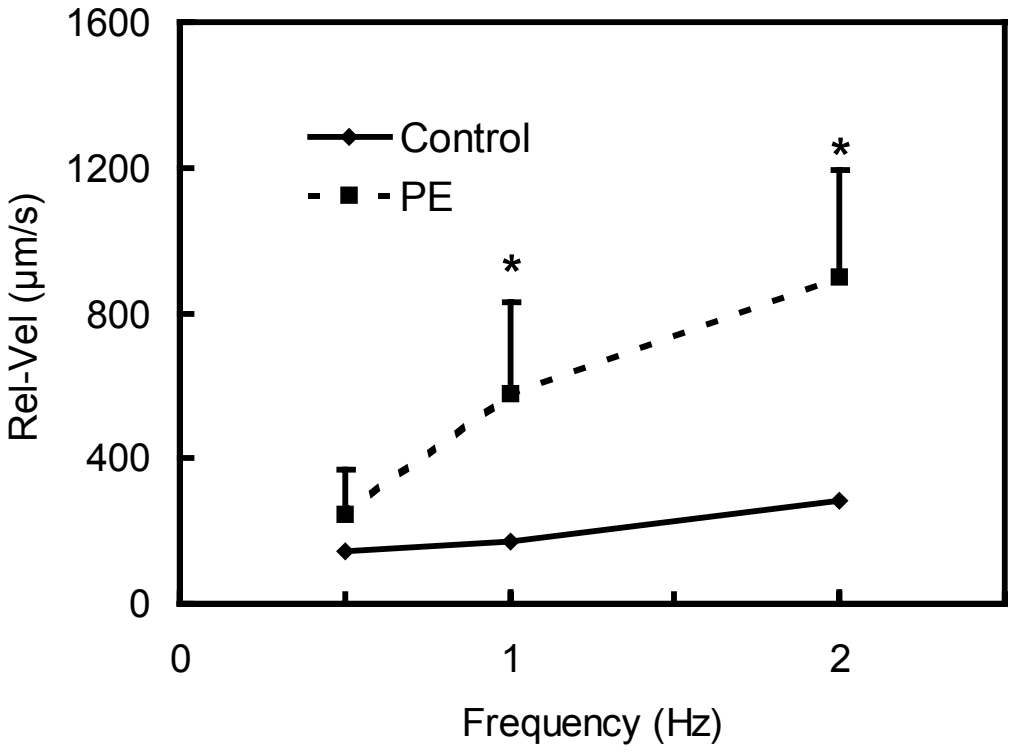


Fig: 4.2.2. The impact of α-adrenoceptor stimulation on relaxation velocity at various beating frequencies. Cells were kept over night under control

conditions (control) or in presence of phenylephrine (PE, 10 µM). Data are means ± S.E.M. (n=24 cells, 4 experiments), * $p<0.05$ vs. control (C).

4.2.3. PE induces SERCA2A expression

Relaxation velocity depends on a fast Ca^{2+} re-uptake in the sarcoplasmatic reticulum. This re-uptake is facilitated by the Ca^{2+} pump called SERCA2A. Therefore, it was tested if PE enhances SERCA2A expression.

Cultured adult ventricular cardiomyocytes were exposed to PE for 24 h. SERCA2A expression after 24 h was determined on the protein level via immunoblotting using antibodies directed against SERCA2A (polyclonal antibody C-20). For loading controls, blots were re-probed with an actin antibody (monoclonal anti-actin clone AC-40). After 24 h SERCA2A protein expression was significantly increased to 181.7 ± 25.1 % as compared to 100 % in controls (Fig.4.2.3.B).

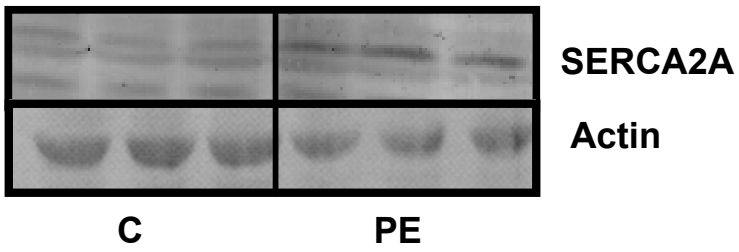


Fig: 4.2.3.A. Effect of α-adrenoceptor stimulation on SERCA2A protein expression. Representative immunoblot indicating increased SERCA2A protein expression in cells treated for 24 h with PE.

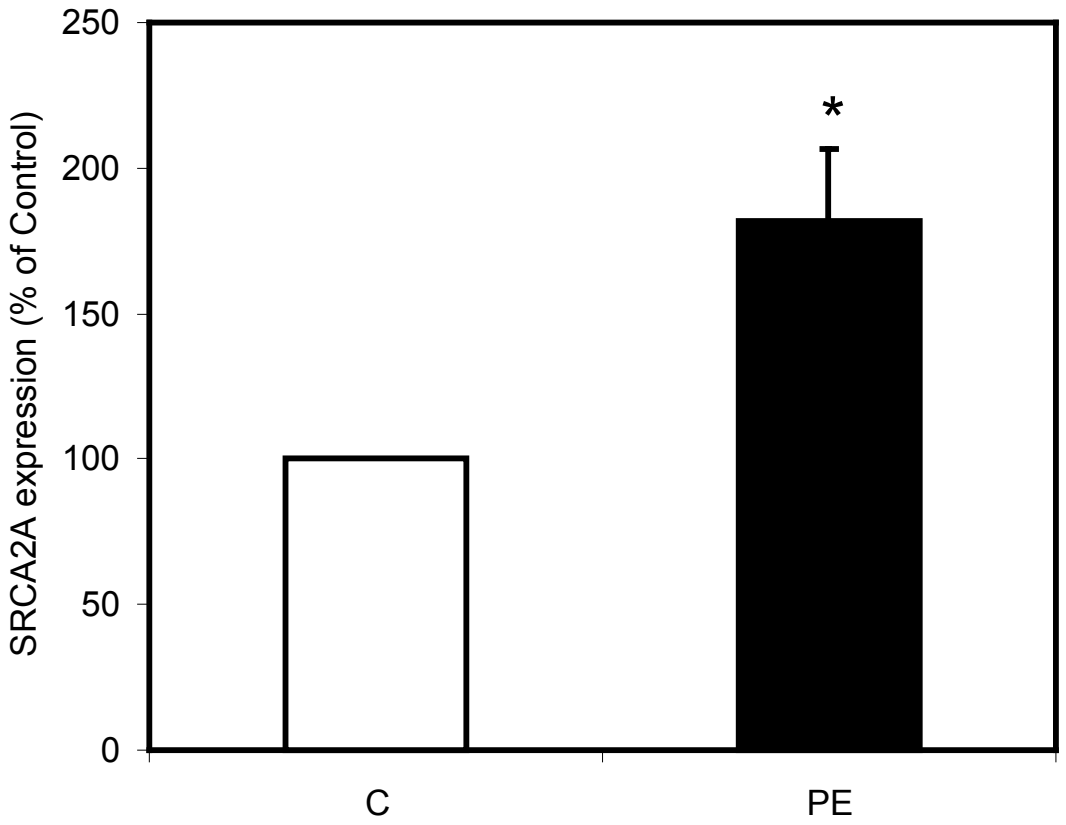


Fig: 4.2.3.B. SERCA2A protein expression. Immunoblots of SERCA2A were quantified. On average, SERCA2A protein expression increased in the presence of phenylephrine after 24 h as compared to control values (C). Data are means ± S.E.M. of n = 4 experiments, * $p<0.05$ vs. control.

In addition to the protein level, mRNA expression of SERCA2A was analysed. Therefore, RNA was isolated at different time points of PE incubation. SERCA2A mRNA expression was quantified by real-time RT-PCR. HPRT was used as a housekeeping gene to normalize sample contents. PE induced SERCA2A mRNA expression after 1.5 h to 125 ± 17 %, and after 4 h to 183 ± 6.5 % as compared to control values. After 24 h up-regulation of SERCA2A mRNA was no more detected (71 ± 9.5 %). This shows that phenylephrine, induced a transient increase in SERCA2A mRNA level, with a peak at 4 h (Fig.4.2.3.C).

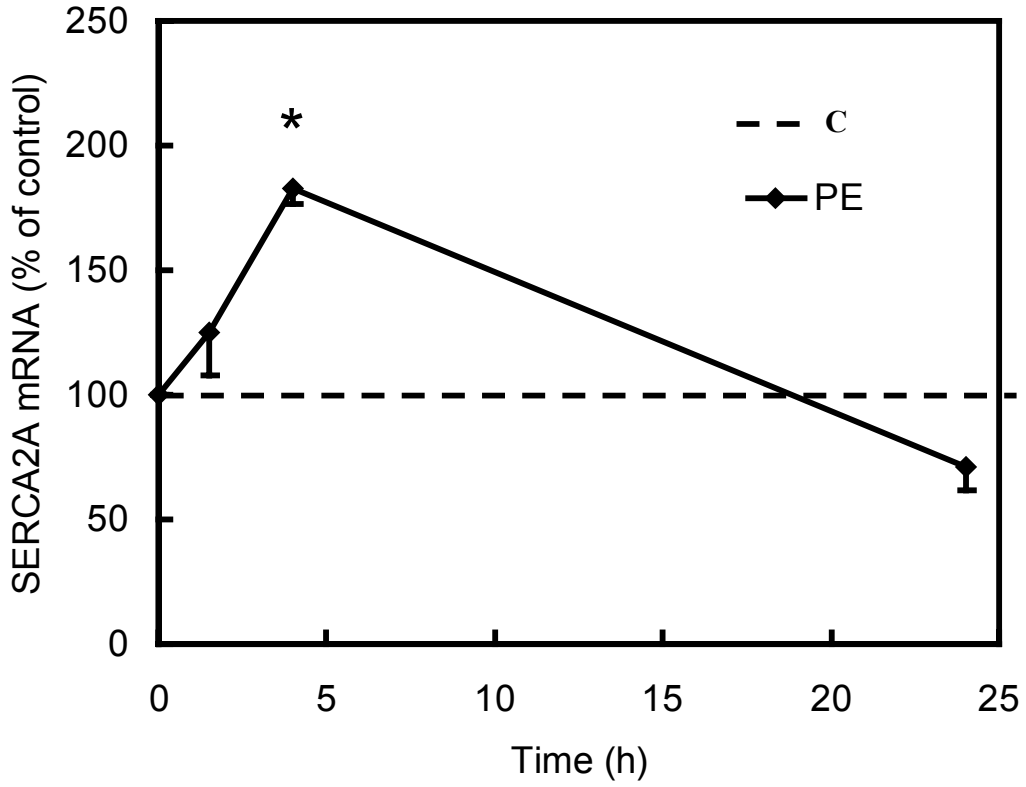


Fig: 4.2.3.C. Effect of α-adrenoceptor stimulation on SERCA2A expression. SERCA2A mRNA expression was quantified by real-time RT-PCR. Data are normalized to HPRT as a housekeeping gene. Cells were kept under control conditions (C) and in the presence of phenylephrine (PE, 10 μM). Data are means ± S.E.M. from n = 4 experiments, * $p<0.05$ vs. control.

4.2.4. Signaling pathway of SERCA2A up-regulation

4.2.4.1. PKC is not involved in SERCA2A up-regulation

Since it is known that PE stimulates PKC in cardiomyocytes, involvement of PKC in SERCA2A up-regulation under PE was determined. Cells were stimulated with PE (10μM) or PE in the presence of the PKC inhibitor Gö6850 (1μM). RNA was extracted after 1.5, 4 and 24 h and real-time PCR was performed. Data were

normalized to HPRT as housekeeping gene. SERCA2A mRNA induction by PE was not influenced by Gö6850. After 4 h SERCA2A mRNA in presence of Gö6850 was still enhanced by PE to 191 ± 8 % (Fig.4.2.4.1.A). The effect of phenylephrine on SERCA2A mRNA expression is therefore, considered to be PKC-independent, because it could not be antagonized by PKC inhibitor Gö6850.

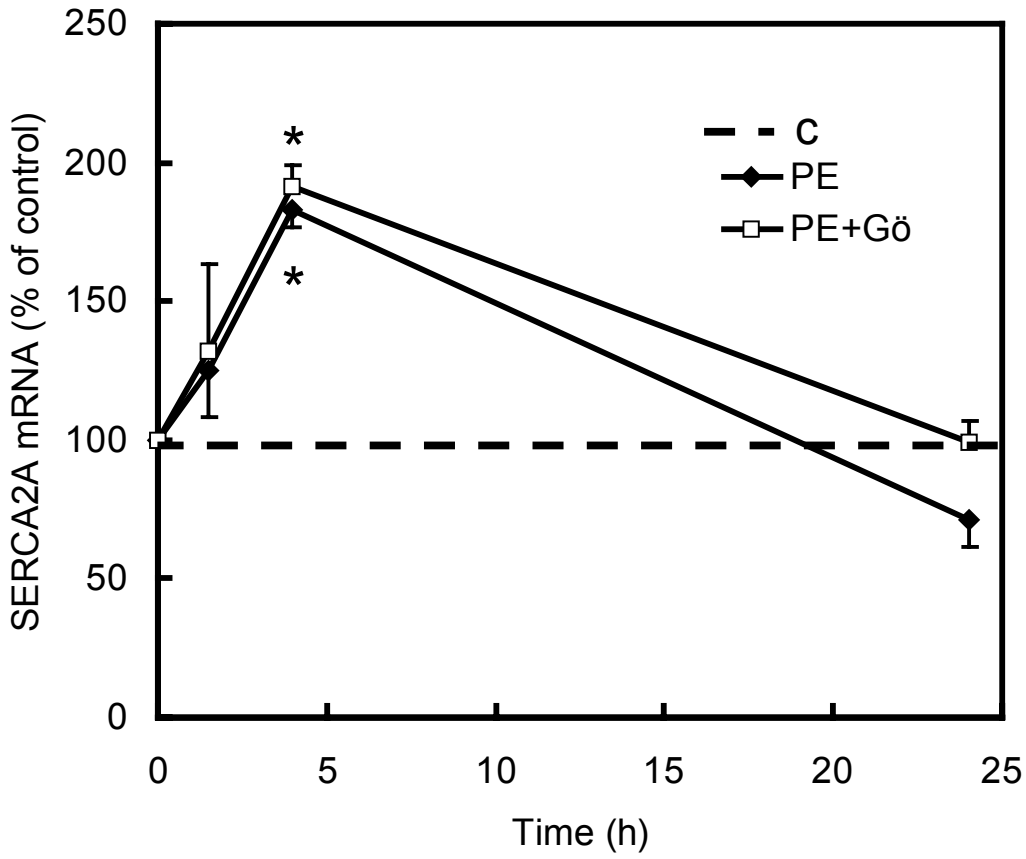


Fig: 4.2.4.1.A. Effect of α-adrenoceptor stimulation on SERCA2A expression. SERCA2A mRNA expression was quantified by real-time RT-PCR. Data are normalized to HPRT as a housekeeping gene. Cells were kept under control conditions (C), in presence of phenylephrine (PE, 10 μM), or PE in co-

presence of the PKC-inhibitor Gö6850 (Gö, 1 µM) for the times indicated. Data are means ± S.E.M. from n = 4 experiments, * p<0.05 vs. control.

Effects of Gö6850 were also tested on the protein level via immunoblotting using antibodies directed against SERCA2A. When cardiomyocytes were incubated with PE or PE in the presence of Gö6850 for 24 h, SERCA2A protein expression was significantly increased in the presence of PE to 181.7 ± 25.1 % as compared to controls. This SERCA2A induction was not reduced by the PKC inhibitor Gö6850 after 24 h (187.2 ± 44.4 %) (Fig.4.2.4.1.C).

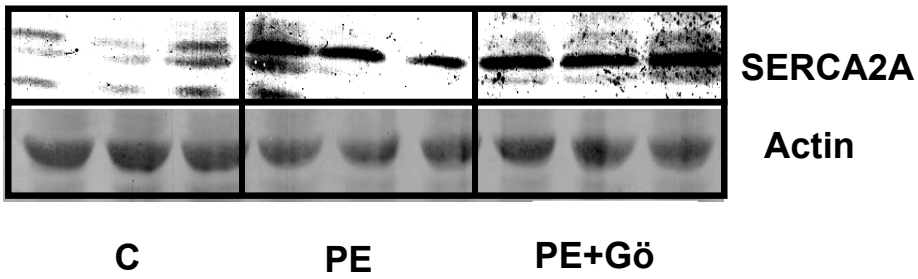


Fig: 4.2.4.1.B. Effect of α-adrenoceptor stimulation on SERCA2A expression. Cells were kept under control conditions (C), in presence of phenylephrine (PE, 10 µM), or PE in co-presence of the PKC-inhibitor Gö6850 (Gö, 1 µM) for 24 h. Representative immunoblots indicating SERCA2A protein (top) and actin (bottom) that was used as a loading control.

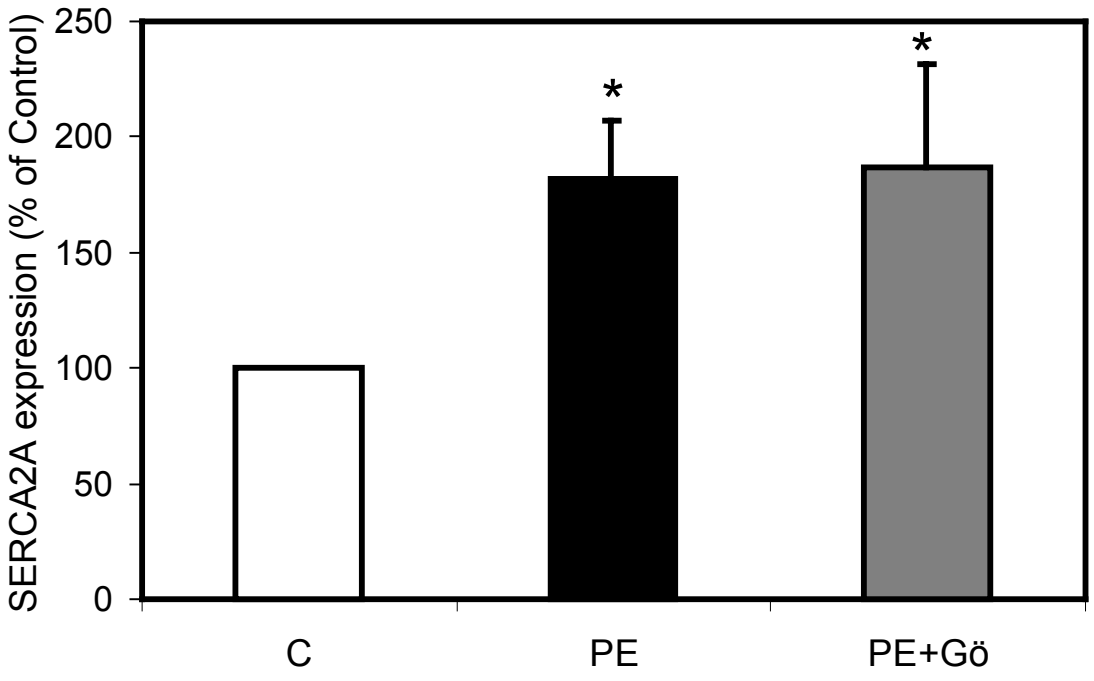


Fig: 4.2.4.1.C. Quantification of immunoblots (SERCA2A protein expression). Cells were treated for 24 h with agonists. Data are means ± S.E.M. of n = 4 experiments, * $p<0.05$ vs. control.

In parallel to the experiments on SERCA2A expression, ^{14}C-phenylalanine incorporation was determined to quantify the rate of protein synthesis. These experiments confirmed earlier observations that the increase of protein synthesis caused by phenylephrine depends on PKC activation. PE increased ^{14}C-phenylalanin incorporation by 50.0 ± 7.5 % compared to control cultures ($P < 0.05$, n = 4 experiments), but not in the presence of Gö6850 (-5.5 ± 5.3 %). These confirmatory experiments indicated that the PKC inhibitor worked in these newly performed experiments and that absence of PKC involvement in SERCA2A induction was not due to failure of the inhibitor.

4.2.4.2. Ca^{2+} / Calcineurin pathway is involved in SERCA2A up-regulation

Based on the experiments described above, the signal transduction pathway leading to SERCA2A up-regulation by phenylephrine was found to be PKC-independent. As has been shown previously, PKC-independent is also an increase in intracellular Ca^{2+} after PE stimulation. Therefore, involvement of the Ca^{2+} / Calcineurin pathway in SERCA2A up-regulation was now studied.

Cells were treated for 24 h with PE or PE in presence of BAPTA (10 μM), a calcium-chelating agent. The effect of phenylephrine on SERCA2A expression could be inhibited by addition of BAPTA. SERCA2A protein expression increased to 258.6 ± 30.4 % in the presence of phenylephrine and decreased to 139.4 ± 21.5 % in the presence of PE and BAPTA as compared to controls (Fig.4.2.4.2.B).

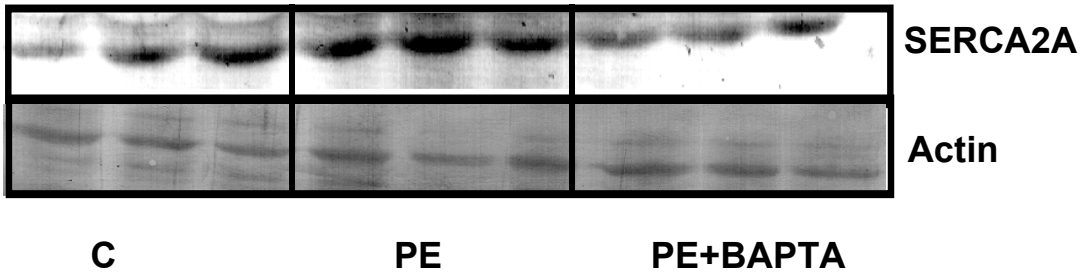


Fig: 4.2.4.2.A. Impact of intracellular calcium on phenylephrine-induced SERCA2A expression. Representative immunoblot indicating SERCA2A protein (top) and actin (bottom) that was used as a loading control. Cells were treated for 24 h with PE or PE in co-presence of BAPTA (10 μM).

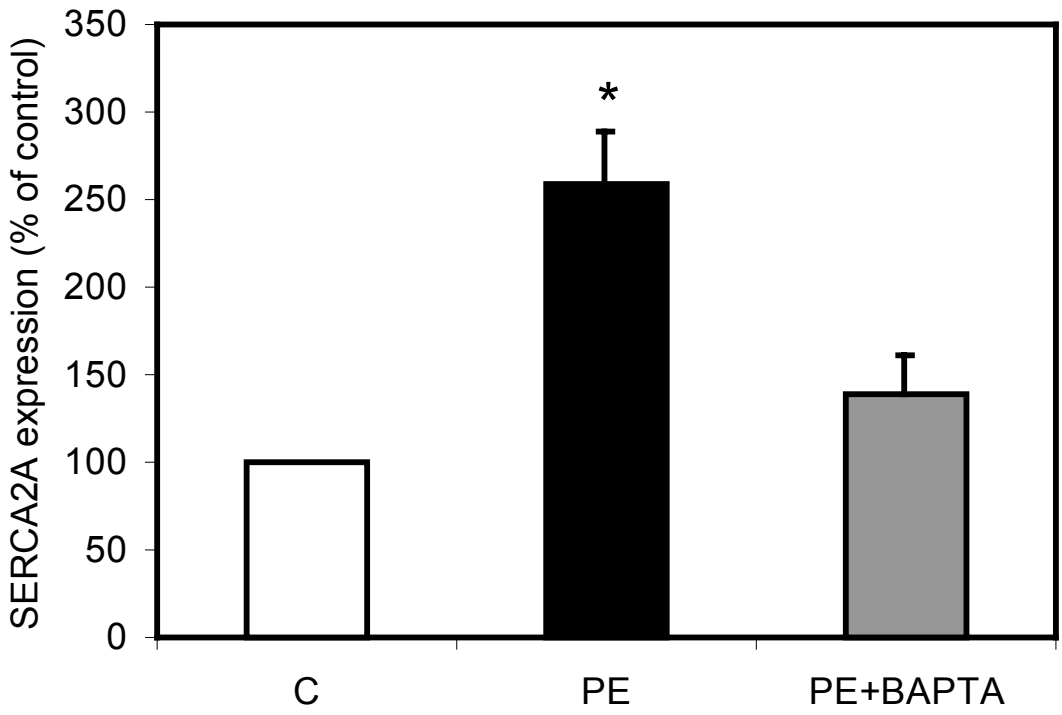


Fig: 4.2.4.2.B. Impact of intracellular calcium on phenylephrine-induced SERCA2A expression. Quantification of immunoblots. Data are means ± S.E.M. from n = 4 experiments, * $p<0.05$ vs. control (C).

Since Ca^{2+} often sequesters with calcineurin in order to convey its function, it was further investigated whether induction of SERCA2A expression depends on calcineurin. Cardiomyocytes were stimulated with phenylephrine in the presence or absence of cyclosporine (1μM), a calcineurin inhibitor. It was confirmed in this set of experiments that phenylephrine increases SERCA2A expression by 179.1 ± 9.1 % as compared to control values. This effect was completely inhibited by cyclosporine (108 ± 6.3 %) (Fig.4.2.4.2.C).

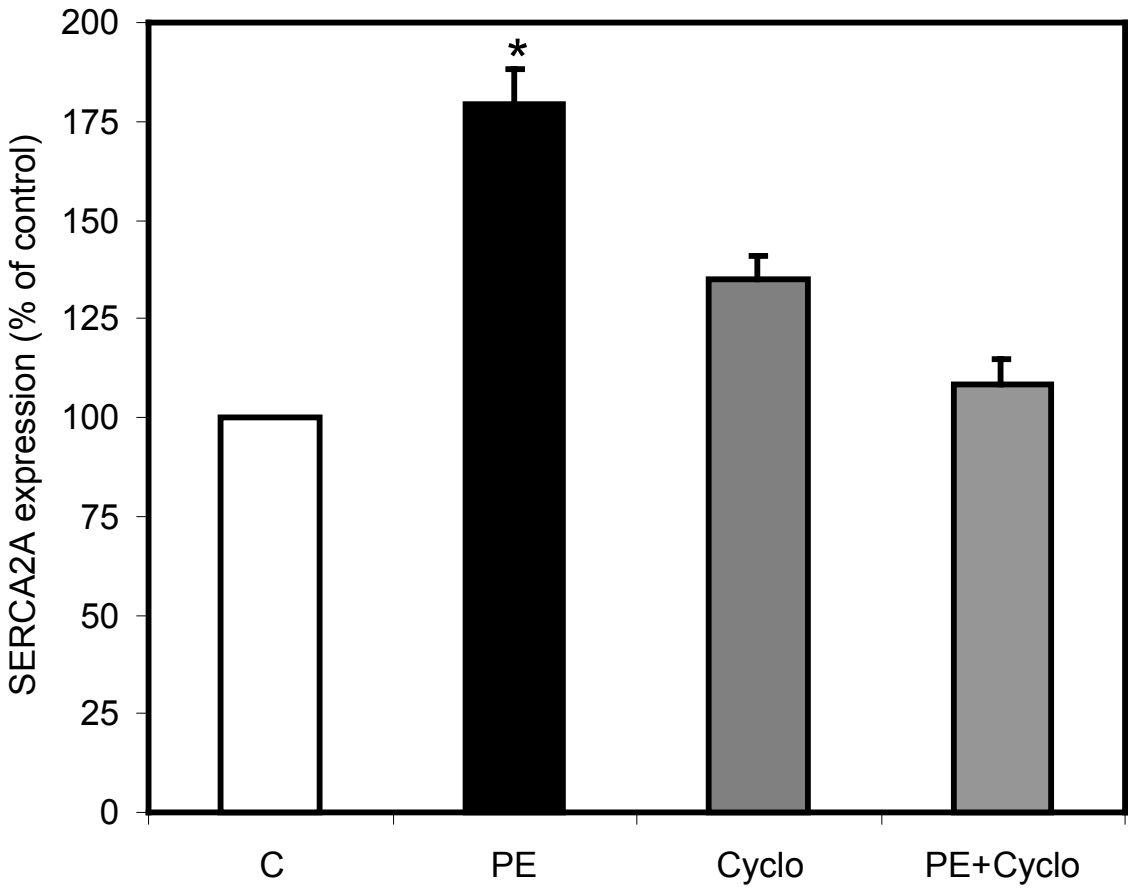


Fig: 4.2.4.2.C. Influence of cyclosporine on SERCA2A expression in cardiomyocytes. Quantitative analysis of SERCA2A expression (normalized to actin) from cells kept over night under control conditions (C), in presence of phenylephrine (PE, 10 µM), cyclosporine (cyclo, 1 µM), or PE in co-presence of cyclo. Data are means ± S.E.M. from n = 4 experiments, * $p<0.05$.

4.2.4.3. NFAT is involved in SERCA2A up-regulation

Since it is known that calcineurin is an activator of the transcription factor NFAT and NFAT has been shown to be induced by PE (Fig.4.1.1.B), it could be assumed that NFAT is also involved in SERCA2A up-regulation. To investigate whether induction of SERCA2A expression depends on NFAT activation, cardiomyocytes were stimulated with PE in the presence or absence of NFAT and Mut-NFAT decoy oligos. It was reconfirmed that PE increased SERCA2A

expression to 135.8 ± 8.4 %. This effect of PE was inhibited by NFAT decoy oligos (100.6 ± 7.2 %). Whereas in the presence of Mut-NFAT decoy oligonucleotides PE still increased SERCA2A expression (145.6 ± 11.9 %) (Fig.4.2.4.3).

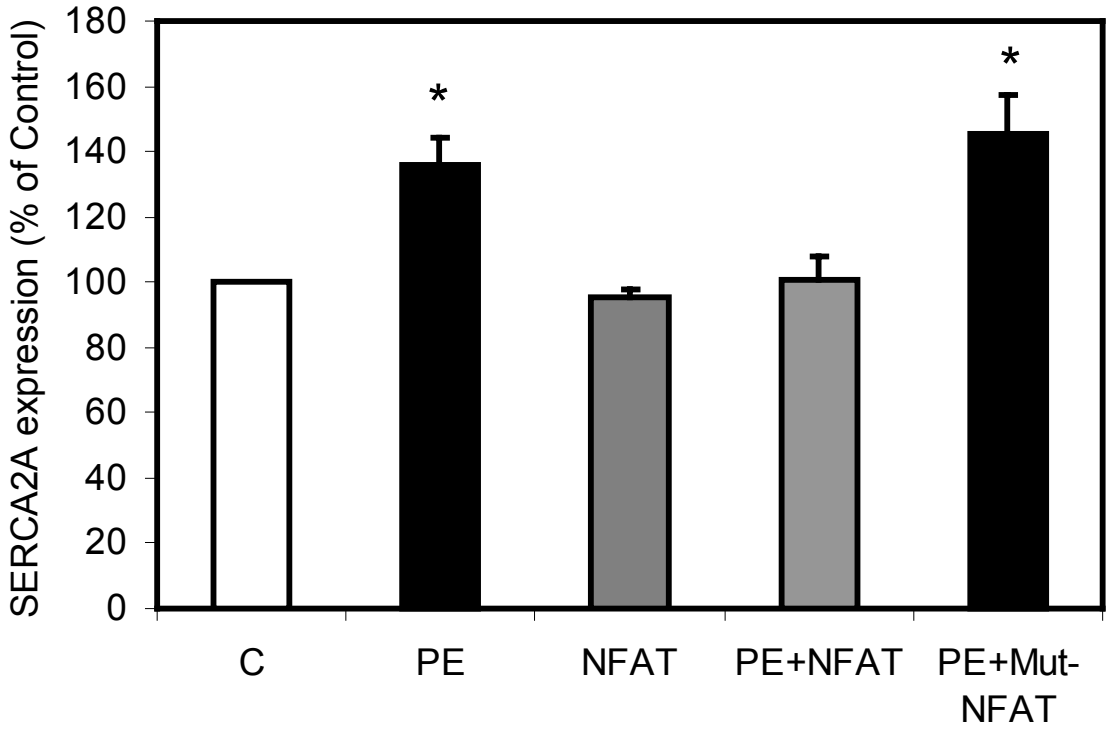


Fig: 4.2.4.3. Quantitative analysis of SERCA2A expression (normalized to actin) from cells kept over night under control conditions (C), kept in presence of phenylephrine (PE, 10 μM), transformed with NFAT or Mut-NFAT oligo decoys (500 nM), or PE in co-presence of NFAT or Mut-NFAT. Data are means ± S.E.M. from n = 4 experiments, * $p<0.05$.

4.2.5. NFAT influences cell shortening under PE

Finally, we confirmed that the afore mentioned changes in SERCA2A expression are of functional relevance. Incubation with 10 μM of PE did not modify the

contractile responsiveness of the cells at 2 Hz as shown in Fig.4.2.1. However, when the cells were co-incubated in the presence of NFAT decoy oligonucleotides (500 nM), contractile responsiveness was significantly depressed at high beating frequencies at 2 Hz (81.2 ± 5.1 % as compared to 100 % controls) (Fig.4.2.5.A).

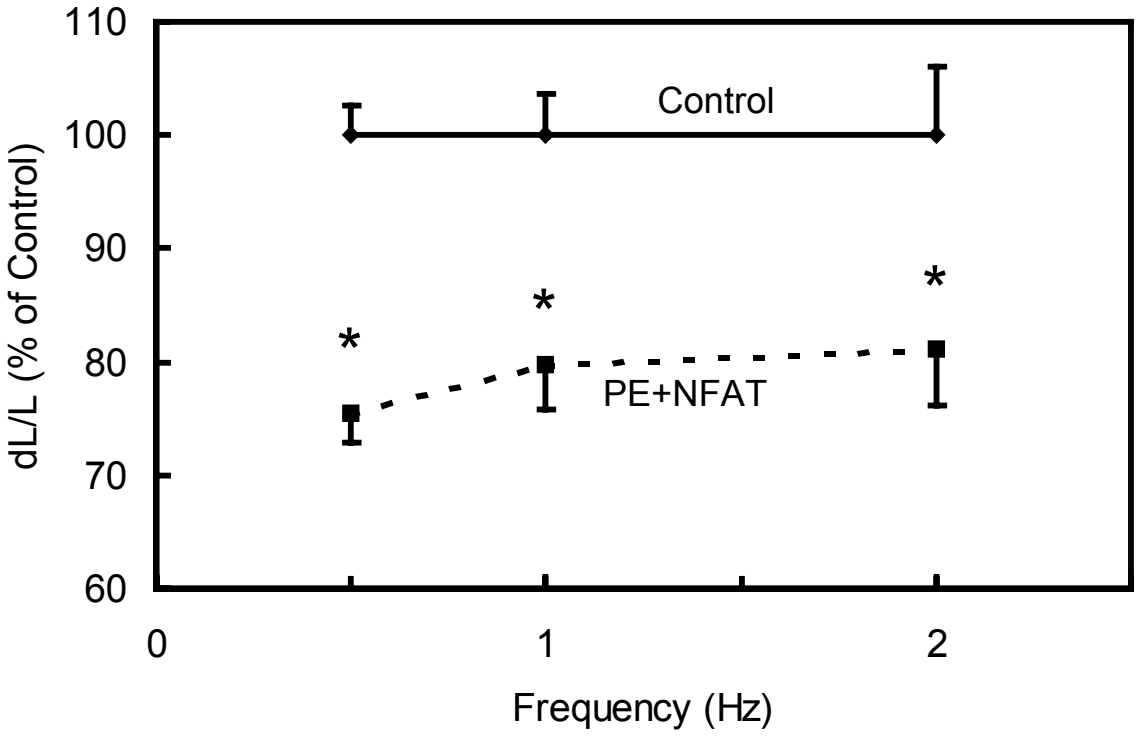


Fig: 4.2.5.A. Impact of NFAT decoy oligos on phenylephrine-induced changes in cell shortening. Cells were kept under control conditions (control) for 48 h or cells transformed with NFAT decoy oligos for 24 h were stimulated with PE (10 µM) for another 24 h. Cell shortening at 0.5, 1.0, and 2.0 Hz is plotted relative to cell shortenings of control cells. Data are means ± S.E.M. from n=36 cells from 4 experiments, * $p<0.05$ vs. control.

When cardiomyocytes were transformed with 500 nM of Mut-NFAT decoy oligonucleotides (void of specific binding site) cell shortening was normal in cells treated with PE at high beating frequencies at 2 Hz (106.6 ± 5.1 % as compared to control) (Fig.4.2.5.B).

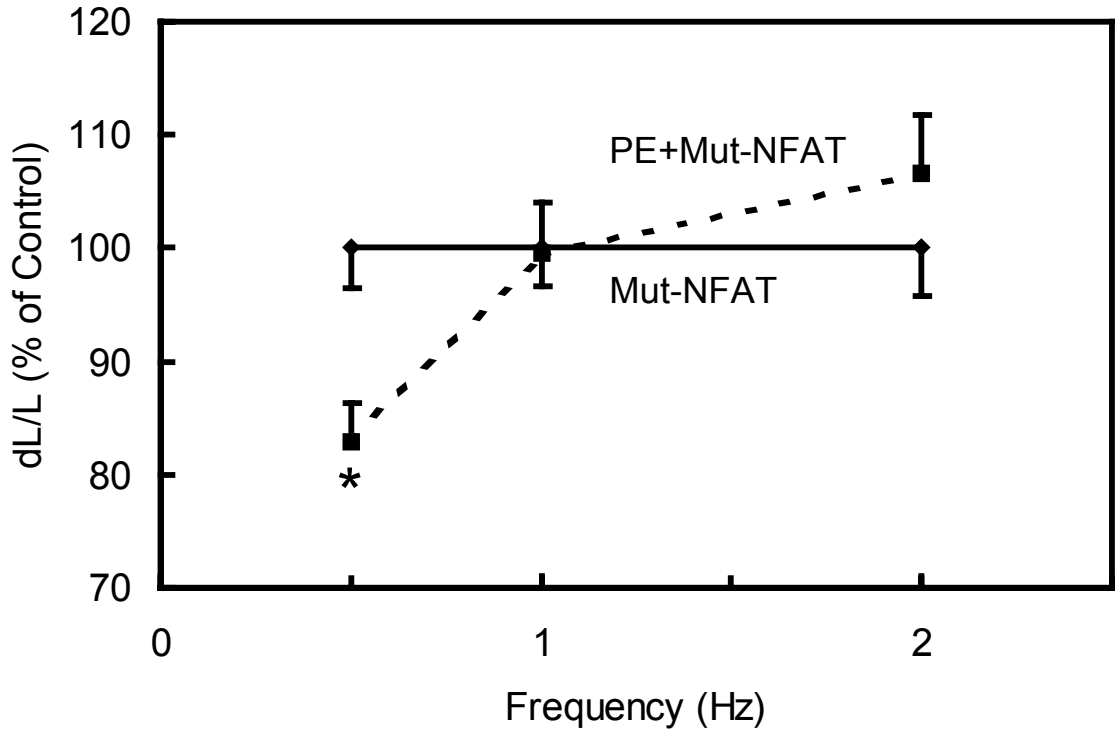


Fig: 4.2.5.B. Impact of Mut-NFAT decoy oligos on PE-induced changes in cell shortening. Cells transformed with Mut-NFAT oligo decoys were kept under control conditions (control) for 48 h or cells transformed with Mut-NFAT oligo decoys for 24 h were stimulated with PE (10 μM) for another 24 h. Cell shortening at 0.5, 1.0, and 2.0 Hz is plotted relative to cell shortenings of Mut-NFAT oligo decoys transformed cells. Data are means ± S.E.M. from n=36 cells from 4 experiments, * $p<0.05$ vs. control.

4.3. Analysis of NFAT activation in ventricular cardiomyocytes of rat under stimulation with β-adrenoceptor agonist ISO

It was now analysed, if stimulation of cardiomyocytes with β-adrenoceptor agonist ISO also influences cell contraction in cardiomyocytes and if a similar pathway as under PE stimulation via NFAT is involved.

4.3.1. NFAT is activated under ISO

Activation of NFAT binding under ISO was determined in retardation gel assays. Cardiomyocytes of adult rat were stimulated with ISO (1 μM) for 2 h. Nuclear extracts of cardiomyocytes were isolated and retardation assay was performed to determine NFAT binding activity. An increased band shift can be detected after stimulation with ISO (Fig.4.3.1.A). NFAT binding was increased to 115.6 ± 2.4 % as compared to controls (Fig.4.3.1.B). This demonstrates that ISO enhances NFAT binding activity in ventricular cardiomyocytes of rat.

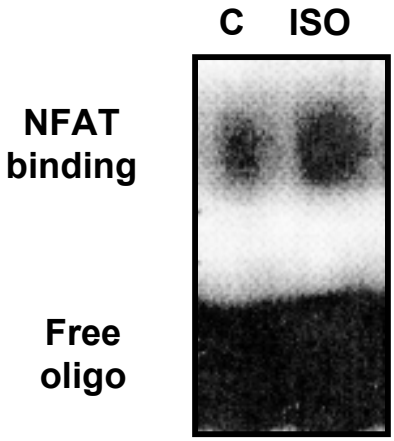


Fig: 4.3.1.A. Stimulation of NFAT binding activity by isoprenaline. This figure represents a retardation gel that indicates enhanced NFAT binding activity in cells treated with isoprenaline (ISO, 1 μM) as compared to untreated controls (C).

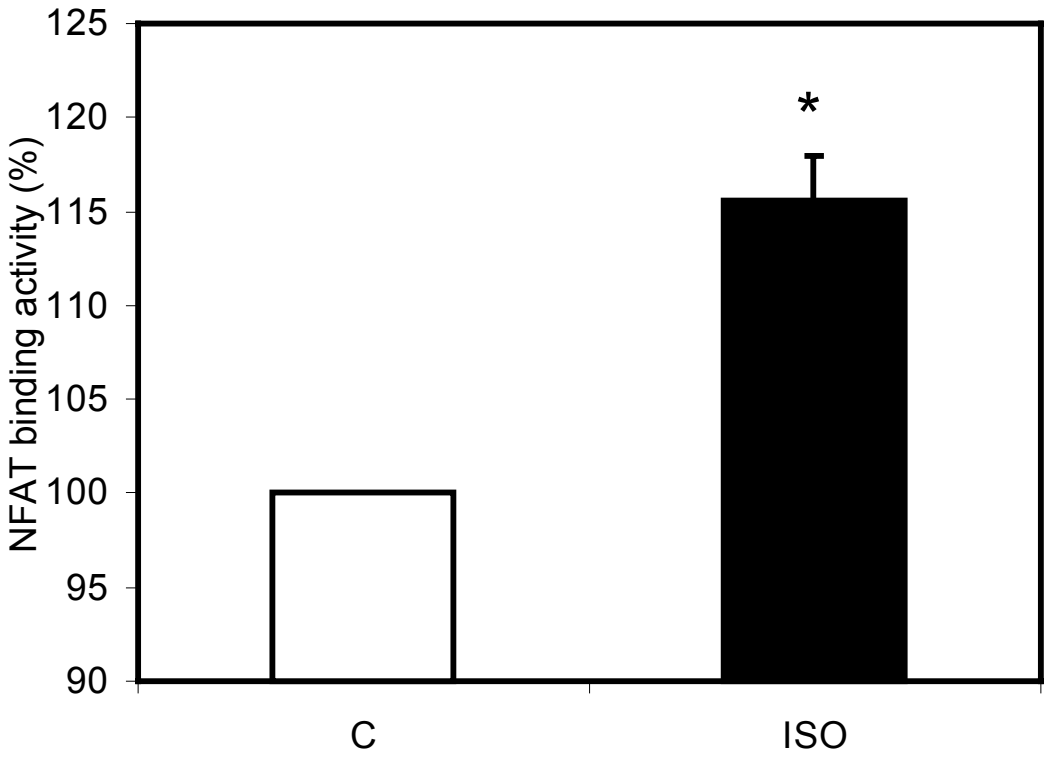


Fig: 4.3.1.B. Stimulation of NFAT-binding activity by isoprenaline. Quantitative analysis of retardation gels indicating enhanced NFAT binding activity in cells treated with isoprenaline (ISO, 1 μM). Data are means ± S.E.M. of n = 6 experiments. * p <0.05 vs. control (C).

4.3.2. Cell shortening under ISO stimulation

Cardiomyocytes were incubated for 24 h in the presence of ISO, then the drug was washed out and cell shortening was analysed using cell-edge detection system. Cells were stimulated at 0.5, 1 or 2 Hz for 1 minute, and every 15 s cell shortening was measured. 24 h exposure of cardiomyocytes to ISO reduced cell shortening at low beating frequency at 0.5 Hz to 88 $\pm$ 3 % as compared to control. However, when beating frequency was increased reduced cell shortening

was no more observed and similar to control when cells were stimulated at 2 Hz (101.9 ± 3.2 %) (Fig.4.3.2.).

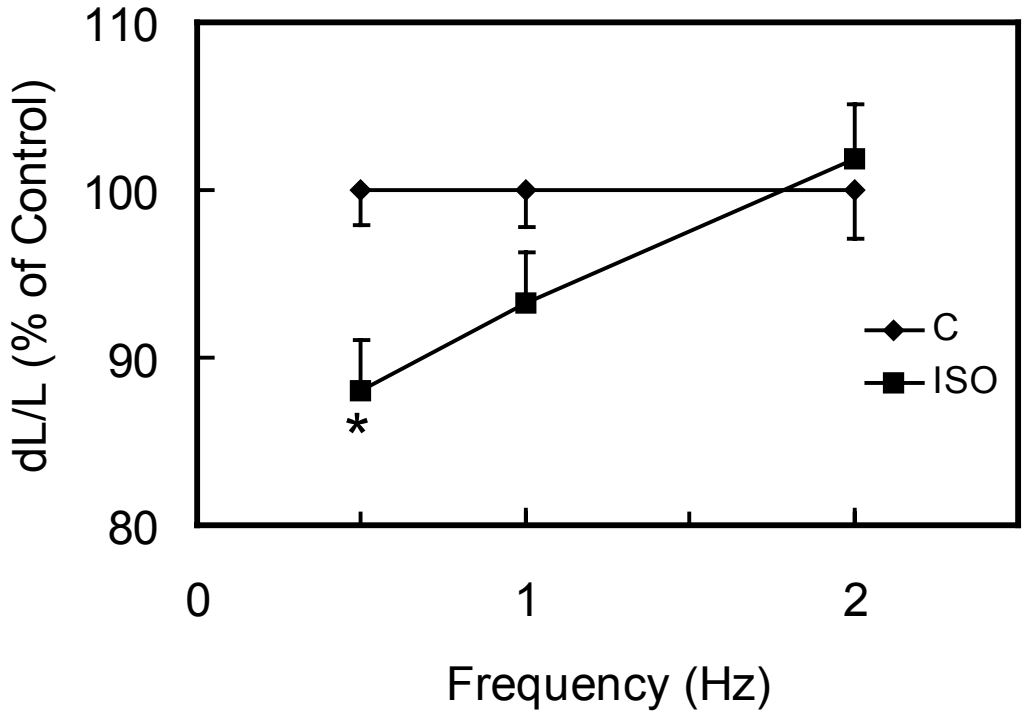


Fig: 4.3.2. The impact of β-adrenoceptor stimulation on cell shortening at various beating frequencies. Cells were kept over night under control conditions (C) or in presence of isoprenaline (ISO, 1 µM). Data are expressed as percent cell shortening relative to the diastolic cell lengths. Data are means ± S.E.M. (n=24 cells, 4 experiments), * $p<0.05$ vs. control.

4.3.3 ISO induces SERCA2A expression

Cultured adult ventricular cardiomyocytes were exposed to ISO for 24 h. SERCA2A expression after 24 h was determined on the protein level via immunoblotting using antibodies directed against SERCA2A (polyclonal antibody C-20). After 24 h SERCA2A protein expression was significantly increased to 132.6 ± 2.8 % as compared to control (Fig.4.3.3.A & B).

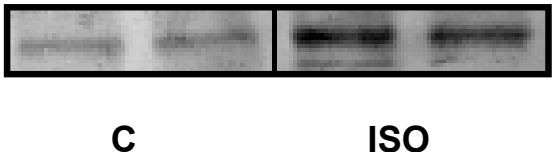


Fig: 4.3.3.A. Effect of β-adrenoceptor stimulation on SERCA2A protein expression. Representative immunoblot indicating SERCA2A protein in cells treated for 24 h with ISO.

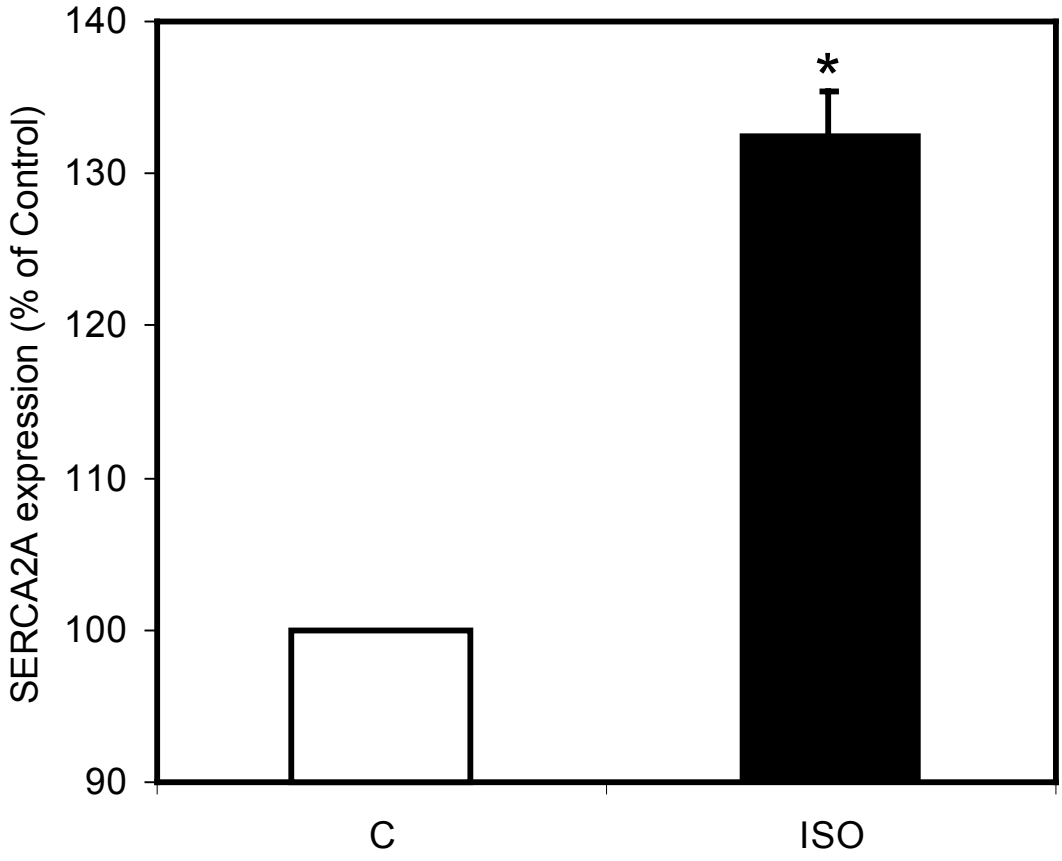


Fig: 4.3.3.B. SERCA2A protein expression. Immunoblots of SERCA2A were quantified. Data are means ± S.E.M. of n = 6 experiments. * $p<0.05$ vs. control (C).

In addition to the protein level, mRNA expression of SERCA2A was analysed. Therefore, RNA was isolated after 4 h of ISO incubation. SERCA2A mRNA expression was quantified by real-time RT-PCR. HPRT was used as a housekeeping gene to normalize sample contents. ISO induced SERCA2A mRNA expression after 4 h to 118 ± 4.1 % as compared to control values. This shows that isoprenaline induced an increase in SERCA2A mRNA level within 4 h (Fig.4.3.3.C).

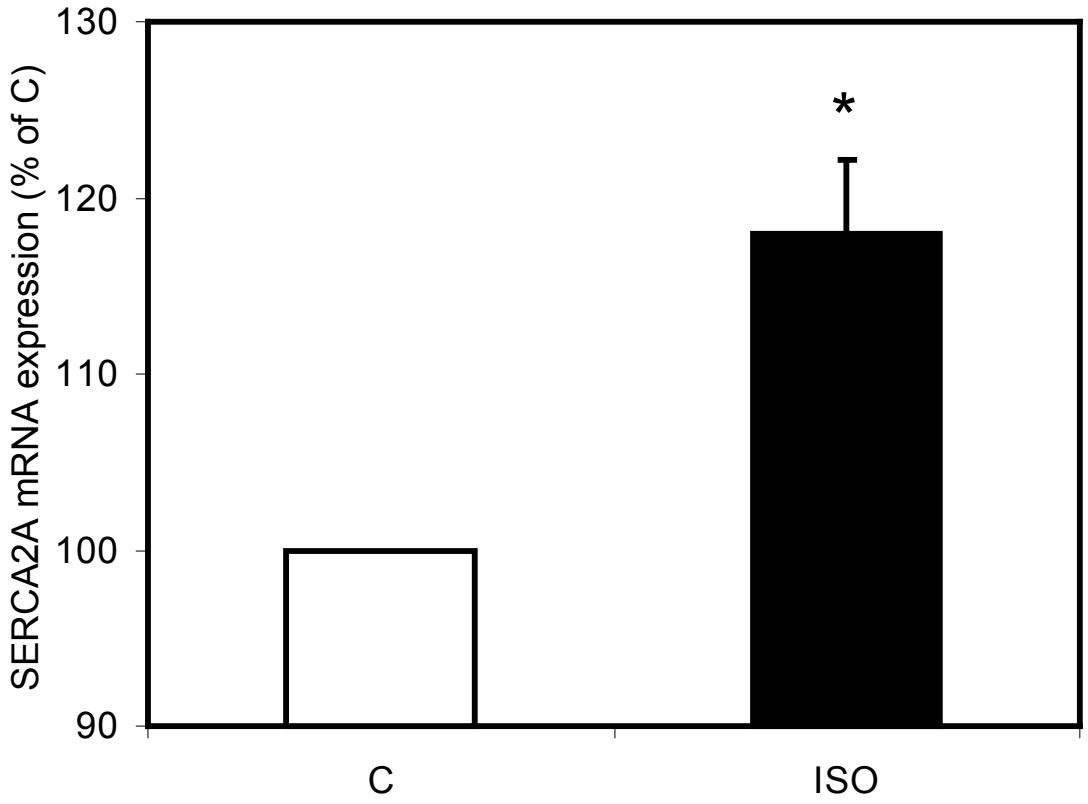


Fig: 4.3.3.C. Effect of β-adrenoceptor stimulation on SERCA2A mRNA expression. SERCA2A mRNA expression was quantified by real-time RT-PCR. Data are normalized to HPRT as a housekeeping gene. Cells were kept under control conditions (C) and in presence of isoprenaline (ISO, 1 µM). Data are means ± S.E.M. from n = 4 experiments. * $p<0.05$ vs. control.

4.3.4. Signaling pathway of SERCA2A up-regulation under ISO

4.3.4.1. NFAT mediates SERCA2A up-regulation under ISO

Cells were stimulated with ISO (1 μM) for 2 h. Then nuclear extracts of cardiomyocytes were isolated and used in retardation assays. For scavenging of NFAT binding, nuclear extracts of cardiomyocytes were pre-incubated with 1 μl of (100 μM) NFAT oligonucleotides (containing the specific binding sequences for NFAT) or 1 μl of (100 μM) Mut-NFAT decoy oligonucleotides (void of specific binding site). Then nuclear extracts were incubated with fluorescence labeled NFAT oligonucleotides, which have NFAT binding sequences. The samples were run on retardation assay gels. These experiments showed that NFAT was activated in cells treated with isoprenaline. This enhanced NFAT activity was reduced by NFAT oligonucleotides but not by Mut-NFAT decoy oligos as compared to control (Fig.4.3.4.1.A), thereby demonstrating specificity of NFAT binding in retardation assays.

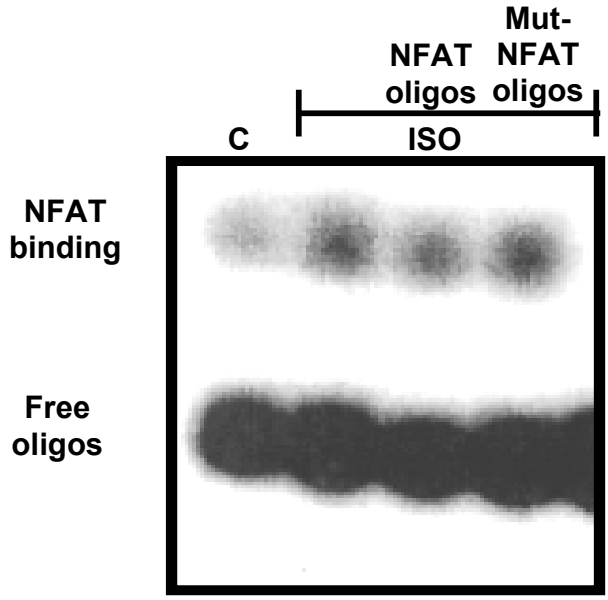


Fig: 4.3.4.1.A. Inhibition of NFAT binding by NFAT oligonucleotides. Retardation gels indicating NFAT binding activity under control conditions (C), and in cells treated with isoprenaline (ISO, 1 μM). When nuclear extracts of ISO

induced cells were pre-incubated with NFAT oligos, NFAT binding was reduced, whereas Mut-NFAT oligo did not influence NFAT binding.

To block NFAT binding intracellular, cells were transformed with NFAT decoy oligos. Therefore, cells were treated for 24 h with ISO (1 μM) in presence or absence of NFAT decoy oligonucleotides (500 nM). SERCA2A protein expression increased to 159.6 ± 5.9 % in the presence of isoprenaline and decreased to 114.4 ± 6.4 % in the presence of ISO and NFAT decoy oligo as compared to controls (Fig.4.3.4.1.C). These results show that enhanced SERCA2A protein expression by ISO was inhibited by NFAT decoy oligos.

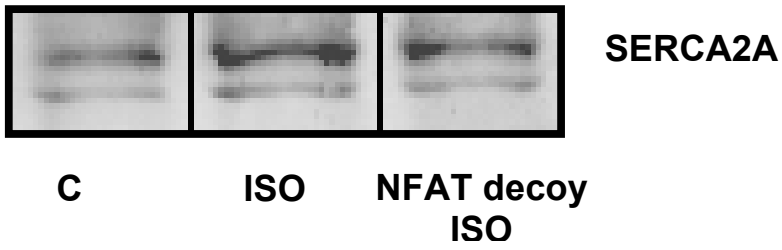


Fig: 4.3.4.1.B. Isoprenaline-induced SERCA2A expression was inhibited by NFAT decoy oligos. Representative immunoblot indicating SERCA2A protein expression. Cells were treated for 24 h with ISO in absence or presence of NFAT decoy oligos (500 nM).

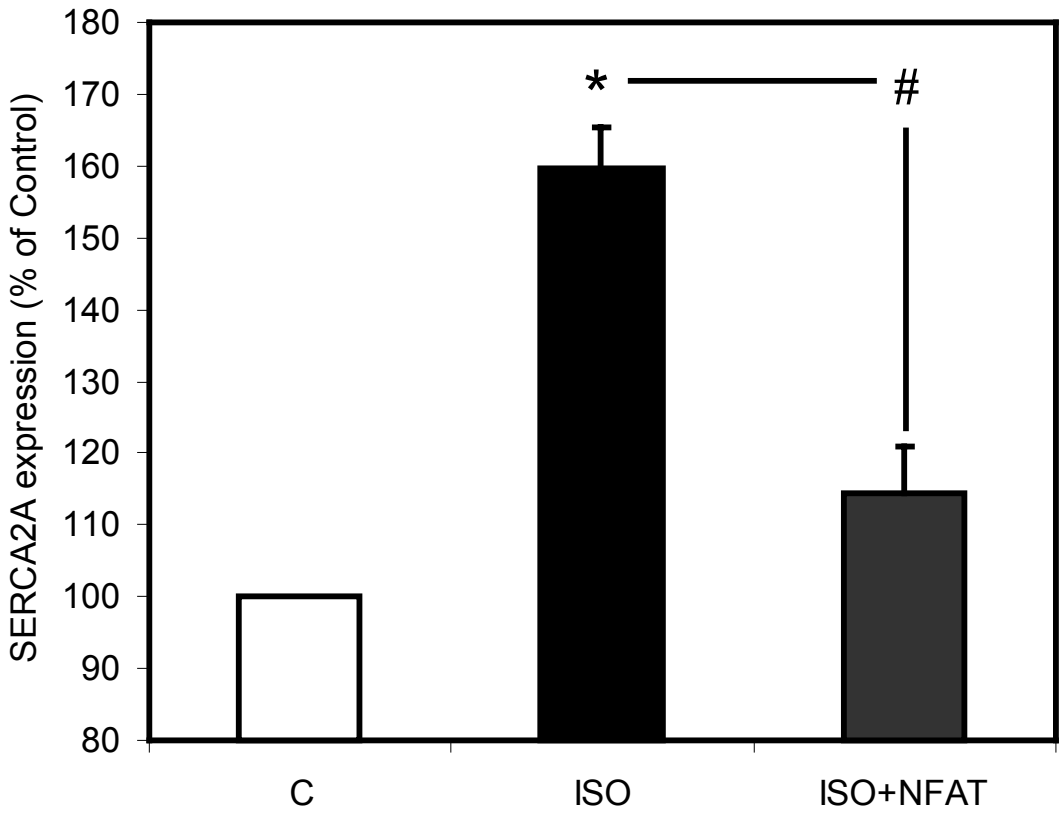


Fig: 4.3.4.1.C. SERCA2A protein expression. Quantification of immunoblots. Data are means ± S.E.M. from n = 6 experiments. * $p<0.05$ vs. control (C).

Besides up-regulation of SERCA2A protein it was further analysed, if SERCA2A mRNA under ISO was also down regulated by NFAT decoy oligos. Cells were stimulated with ISO (1 μM) or ISO in the presence of the NFAT decoy oligonucleotides (500 nM) for 4 h. Data were normalized to HPRT as housekeeping gene. Isoprenaline induced SERCA2A mRNA up-regulation to 118 ± 4.1 %. This increase was totally abolished in cardiomyocytes transformed with NFAT decoy oligos (81.5 ± 6.9 %) (Fig.4.3.4.2.). Therefore, NFAT is considered to be involved in SERCA2A up-regulation on the mRNA and protein level.

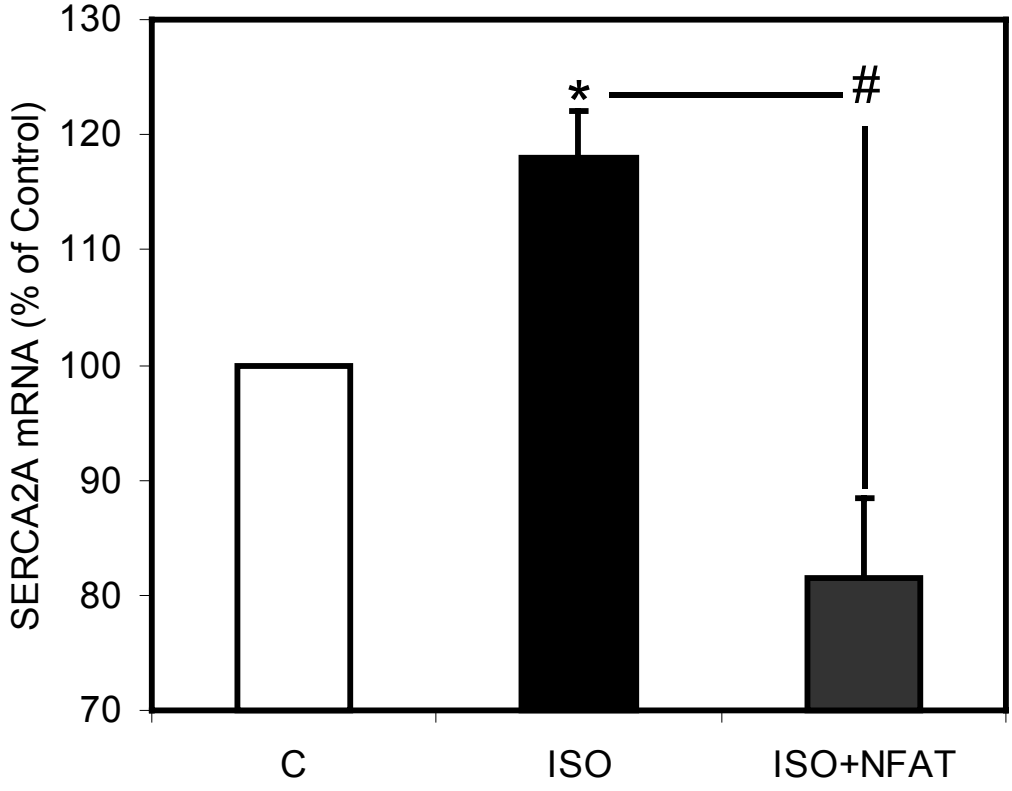


Fig: 4.3.4.2. Effect of β-adrenoceptor stimulation on SERCA2A expression. SERCA2A mRNA expression was quantified by real-time RT-PCR. Data are normalized to HPRT as a housekeeping gene. Cells were kept under control conditions (C), in presence of isoprenaline (ISO, 1 µM), or ISO in co-presence of the NFAT decoy oligonucleotide (NFAT decoy, 500 nM) for 4 h. Data are means ± S.E.M. from n = 4 preparations. * $p<0.05$ vs. control. # = $p<0.05$ vs. ISO.

4.3.4.3. AP-1 is involved in SERCA2A up-regulation

Under ISO stimulation induction of AP-1 has been described (Taimor et al., 2004). Since NFAT and AP-1 are co-operating transcription factors it was analysed if AP-1 is also involved in SERCA2A up-regulation. Therefore, cardiomyocytes were transformed with CRE decoy oligos which can intracellular

scavenge AP-1 or with scrambled decoy oligos (SCR) that contain no specific AP-1 binding site. Cells were treated for 24 h with ISO (1 μM) in the presence of CRE (250 nM) or SCR decoy oligos (250 nM). SERCA2A protein expression increased to 113.6 ± 3.8 % in the presence of SCR decoy oligos (250 nM) but was decreased to 98.6 ± 21.5 % in the presence of CRE decoy oligos as compared to controls (Fig.4.3.4.3.B). The effect of isoprenaline on SERCA2A expression could be inhibited by addition of CRE decoy oligos (250 nM) and is therefore AP-1-dependent.

Fig: 4.3.4.3.A. Involvement of AP-1 in SERCA2A expression under stimulation of β-adrenoceptor ISO. Cells were kept under control conditions (C), or ISO (1 μM) in co-presence of SCR (250 nM) or CRE decoy oligos (250 nM) for 24 h. Representative immunoblot indicating SERCA2A protein expression.

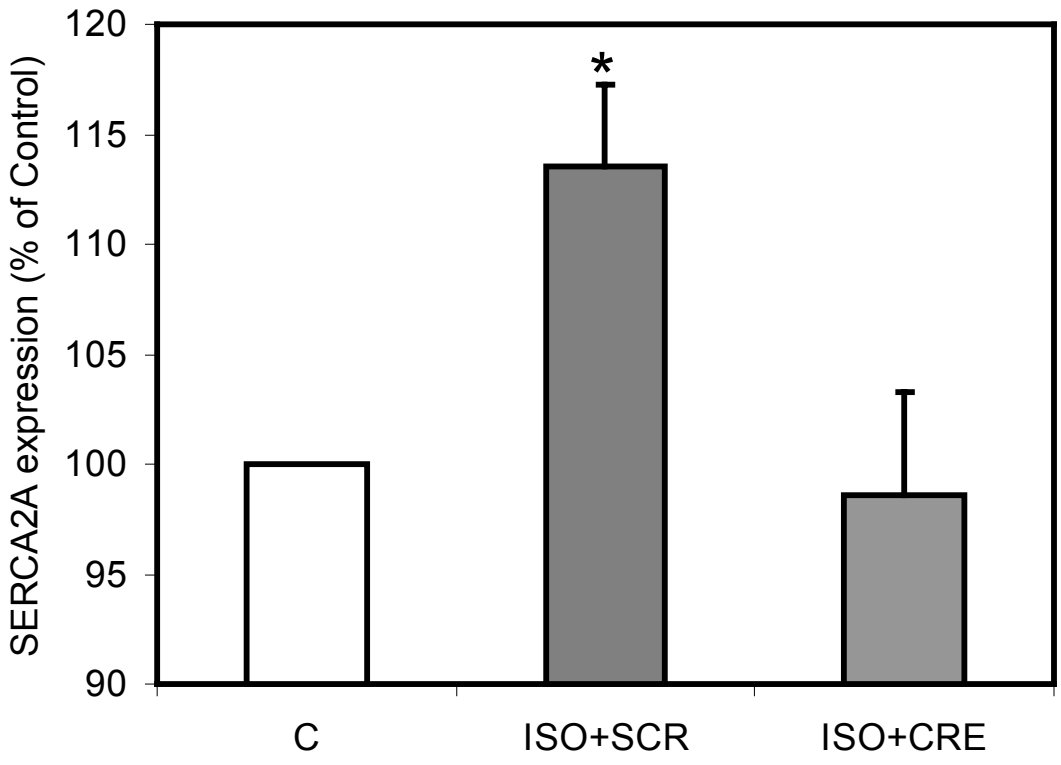


Fig: 4.3.4.3.B. Quantification of immunoblots of SERCA2A protein expression. Cells were treated for 24 h with ISO in presence of decoy oligos. Data are means ± S.E.M. of n = 6 experiments. * $p<0.05$ vs. control.

4.3.5. NFAT is involved in reduced cell shortening under ISO

Finally, we confirmed that the afore mentioned changes in SERCA2A expression are of functional relevance for cell contraction as shown in Fig.4.3.2. Incubation with 1 μM of ISO did not modify the contractile responsiveness of the cells at 2 Hz however, when the cells were co-incubated with NFAT decoy oligonucleotides (500 nM), contractile responsiveness was significantly depressed at high beating frequencies at 2 Hz to 88 ± 3.1 % as compared to 100 % in cells treated with ISO alone (Fig.4.3.5.A).

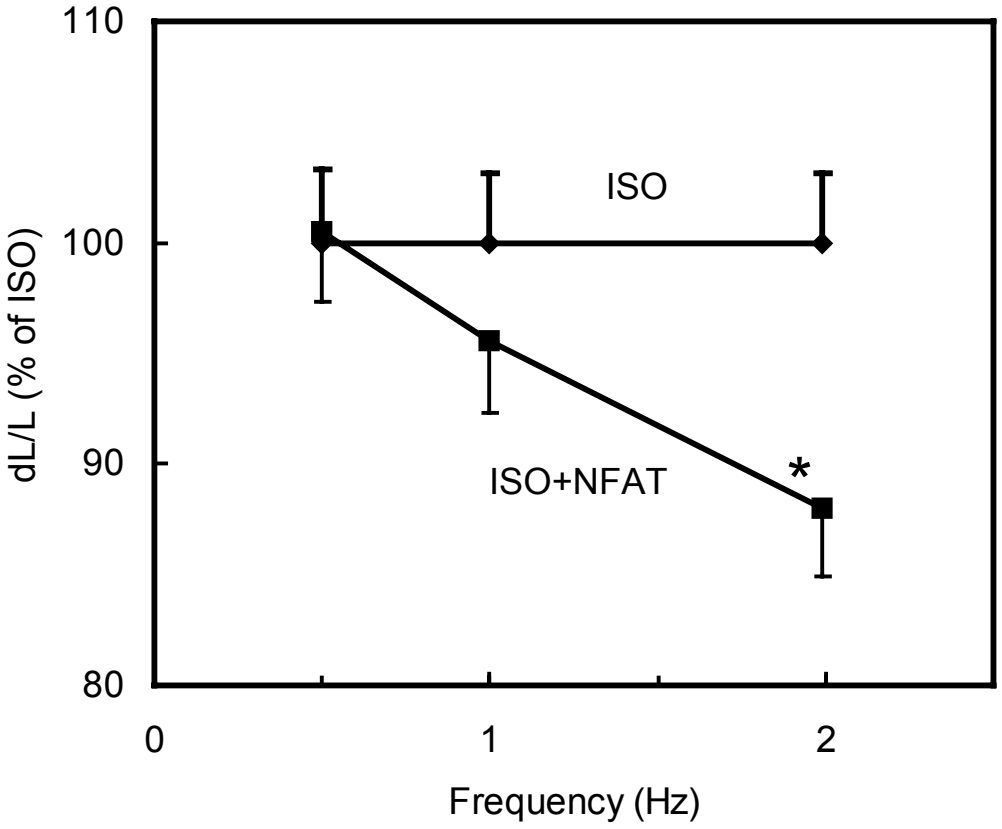


Fig: 4.3.5.A. Impact of NFAT decoy oligos on isoprenaline-induced changes in cell shortening. Cells treated with ISO were kept under control conditions (control) for 48 h or cells transformed with NFAT decoy oligos for 24 h were stimulated with ISO (1 µM) for another 24 h. Cell shortening at 0.5, 1.0, and 2.0 Hz is plotted relative to cell shortenings of ISO treated cells. Data are means ± S.E.M. from n=36 cells from 4 experiments. * $p<0.05$ vs. control.

When cardiomyocytes were transformed with 500 nM of Mut-NFAT decoy oligonucleotides (void of specific binding site) cell shortening was normal in cells treated with ISO at high beating frequencies at 2 Hz (96.3 ± 2.7 %) as compared to ISO treated cells as control (Fig.4.3.5.B).

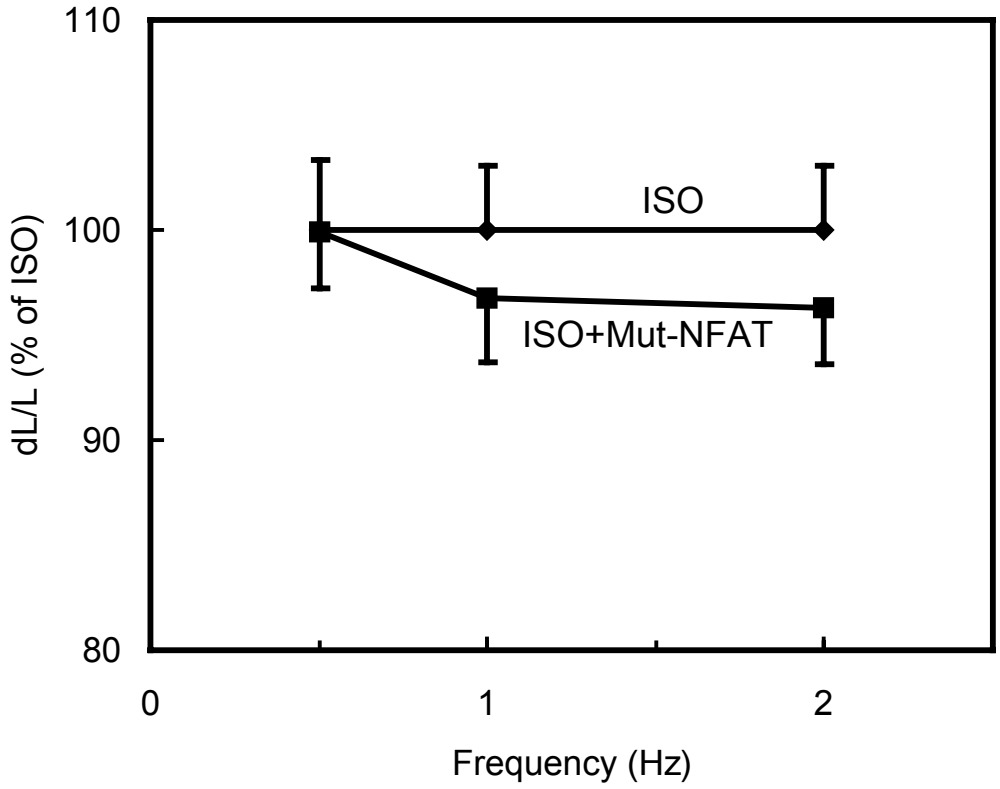


Fig: 4.3.5.B. Impact of Mut-NFAT decoy oligos on isoprenaline-induced changes in cell shortening. Cells were kept under ISO for 48 h as control conditions (control) or cells transformed with Mut-NFAT decoy oligos for 24 h were stimulated with ISO (1 μM) for another 24 h. Cell shortening at 0.5, 1.0, and 2.0 Hz is plotted relative to cell shortenings of ISO treated cells as control. Data are means ± S.E.M. from n=36 cells from 4 experiments. * $p<0.05$ vs. control.

5. Discussion

From previous studies it is known that α-adrenoceptor stimulation by phenylephrine but not the β-adrenoceptor stimulation by isoprenaline induces hypertrophy in freshly isolated adult rat cardiomyocytes (Schlüter and Piper, 1992). The functional consequences of adrenergic stimulation on cardiomyocyte contraction have not been analysed yet.

The main findings of this study are that PE or ISO stimulation of cardiomyocytes decreases cell shortening at 0.5 Hz, but not at high beating frequencies (2 Hz). This normal cell shortening at high beating frequencies can be explained by induction of SERCA2A expression under PE or ISO stimulation in adult rat ventricular cardiomyocytes. The induction of SERCA2A expression is mediated via activation of calcineurin/NFAT pathway. And this SERCA up regulation influences cell shortening since decreased cell shortening in myocytes, transformed with NFAT decoy oligonucleotides, is also present at 2 Hz under PE or ISO stimulation. Interestingly, induction of hypertrophic growth under α-adrenoceptor stimulation was independent of NFAT activation.

5.1. Role of NFAT in cardiomyocyte hypertrophy

In transgenic mice containing an NFAT-dependent luciferase reporter gene it was demonstrated that calcineurin/NFAT signaling is constitutively up regulated throughout a time course of pressure overload hypertrophy, as well as in the failing mouse heart following myocardial infarction (Molkentin JF, 2004). As discussed in the introduction, transgene-mediated over expression of NFAT in the mouse heart antagonized the cardiac hypertrophic response (Antos et al., 2002; Sanbe et al., 2003). Subsequently studies in several transgenic animal models have supported the initial observation that activation of the calcineurin/NFAT signal transduction pathway is both necessary and sufficient for this hypertrophic response (Molkentin JF, 2004). However, there are also reports of transgenic models with genetic loss of calcineurin that blocks mechanical overload induced skeletal muscle fiber type switching but not hypertrophy (Parsons et al., 2004; Pu et al., 2003). Therefore, importance of

calcineurin/NFAT signaling for hypertrophy is not yet clear and a role for NFAT in cardiac hypertrophy under physiological conditions is less well established. There are no confirmed data, which demonstrate that hypertrophic growth in all systems depends on activation of calcineurin/NFAT pathway.

My results indicate that stimulation of α- or β- adrenoceptor agonists significantly activate the calcineurin/NFAT signal transduction pathway in adult cardiomyocytes from rat. In this respect this study confirms earlier reports on neonatal cardiomyocytes in which NFAT activation upon adrenergic stimulation was demonstrated (Molkentin, 2004; Pu et al., 2003; Zobel et al., 2002). In contrast to neonatal cardiomyocytes, I did not find functional involvement of the calcineurin/NFAT pathway in hypertrophic growth of cardiomyocytes. This conclusion is based on the following finding: Transformation of cardiomyocytes with NFAT decoy oligonucleotides did not reduce the PE-induced hypertrophic growth in cardiomyocytes, neither on the level of rate of protein synthesis nor on the level of cross sectional area. So NFAT decoy oligonucleotides did not impair the effect of phenylephrine on protein synthesis and cell size. Therefore, it was concluded that NFAT activation is not involved in the induction of hypertrophy induced by α-adrenoceptor PE stimulation in adult rat cardiomyocytes. Reasons for these different findings compared to neonatal cardiomyocytes or transgenic mice may be the different signaling in cardiomyocytes from diverse differentiation states or the high artificial over-expression of the transgene in mice.

5.2. SERCA2A as a downstream target of the calcineurin/NFAT pathway

It has been shown in previous studies that PE increases intracellular free calcium concentrations but this is not related to hypertrophy (Schäfer et al., 2002; Stengl et al., 1998; De Jonge et al., 1995). An elevation of intracellular calcium will lead to an activation of calcineurin. In most immune-system cells calcineurin regulates the activity of a number of downstream targets including the transcription factor NFAT (Rao et al., 1997). Therefore, NFAT activation can be regulated by calcium/calcineurin pathway. Since calcium signaling under PE has been shown to be independent of hypertrophic growth, it was proposed that NFAT pathway

might influence the cell function under adrenoceptor stimulation. As a down-stream target of calcium/calcineurin/NFAT signaling SERCA2A up-regulation was identified in this study. I have shown that SERCA2A expression was increased at mRNA level after 4 h incubation with PE or ISO and at protein level after 24 h incubation of cardiomyocytes at 37°C. By using BAPTA (calcium blocker) cyclosporine (calcineurin inhibitor) or transformation of cardiomyocytes with decoy oligonucleotides directed against NFAT, PE-induced SERCA2A expression was inhibited. Similar to this response ISO-induced SERCA expression was also blocked by decoy oligonucleotides.

These findings are related to a recent report, which shows a linkage between NFAT and SERCA promoter activity in neonatal rat cardiomyocytes (Vlasblom et al., 2004). These authors demonstrated by the use of 2,3-butanedion monoxime (BDM), which arrests cell contractions, that mechanical unloading increases SERCA expression in a calcineurin/NFAT-dependent way. By using a co-transfection system of cardiomyocytes they also showed activation of SERCA promoter activity by calcineurin/NFAT. My findings that PE or ISO activates NFAT and increases SERCA2A expression is related to these observations. Furthermore, it was also shown previously on transgenic calcineurin model that SERCA expression could be increased on the protein level in a calcineurin-dependent way (Chu et al., 2002). Thus, in agreement with my findings there are indications through-out the literature that the calcineurin/NFAT pathway is positively linked to SERCA expression.

So in my study it was demonstrated that α- or β-adrenoceptor stimulation, calcineurin/NFAT activation and SERCA expression are linked with each other. This is confirmed by following findings:

1. NFAT activation and SERCA expression was induced by stimulation of α- or β-adrenoceptors.
2. Cyclosporine, an inhibitor of calcineurin, or NFAT decoy oligonucleotides, that can inhibit the NFAT could attenuate SERCA expression under adrenergic stimulation.

5.3. SERCA expression and cellular function

A decrease in SERCA2A expression and/or activity seems to be a major defect responsible for impaired function of the failing heart. SERCA2A levels and activity were decreased and severe contractile dysfunction was evident in both conditions of failing cardiac myocytes and intact animal hearts subject to experimental heart failure (Del Monte et al., 1999; Hajjar et al., 1998; Miyamoto et al., 2000). Over expression of SERCA2A by gene transfer in vivo restores both systolic and diastolic dysfunction to normal levels (Periasamy & Huke, 2001). Transgenic mice over expressing SERCA2A develop a lower rate of mortality with improved contractility compared to non-transgenic littermates at the onset of early heart failure in animals with biomechanical overload (Ito et al., 2001). The chronic transgenic expression of SERCA2A in mice enhances survival and systolic performance in vivo and preserves contractile reserve and the capacity to increase SR Ca^{2+} load at high work states in isolated myocytes. Del Monte et al. (1999) showed that over expression of SERCA2A in human ventricular myocytes from patients with end-stage heart failure can increase SERCA pump activity and enhance contraction and relaxation velocity. The negative frequency response was normalized in cardiomyocytes over expressing SERCA2A. Thus in several models importance of SERCA for cardiomyocyte contraction and heart function have been shown.

In the cell model investigated in this study, cells required an up-regulation of SERCA to maintain normal contractile responsiveness at high beating frequencies under α- or β-adrenoceptor stimulation. This conclusion is based on experiments in which cardiomyocytes transformed with NFAT decoy oligonucleotides displayed depressed contractile responsiveness under α- or β-adrenoceptor stimulation. Under both conditions, up-regulation of SERCA was also inhibited. The data suggest that up-regulation of SERCA is an early response of cardiomyocytes due to adrenergic stimulation and that this improves cardiomyocyte contractile function.

My functional findings on single cell shortening and the interaction with SERCA expression are in agreement with previous work on transgenic mice. As already

known from published data, a moderate increase in SERCA expression was found to be sufficient to increase maximal relaxation velocity (He et al., 1997). Relaxation velocity depends on calcium re-uptake by sarcoplasmic reticulum and this reuptake is regulated by SERCA2A. Thus the increase in SERCA2A expression is responsible for the enhancement of relaxation velocity at high beating frequency in ventricular cardiomyocytes, and this results in normal cell shortening at high beating frequency of 2 Hz.

The long-term outcome of hypertrophic response cannot be predicted from this study. However, the effect of α-adrenoceptor stimulation on SERCA expression is transient. This is suggested by the data on SERCA mRNA expression: SERCA mRNA expression was measured by real-time PCR after exposure of cardiomyocytes to PE for 1.5, 4 and 24 h. A transient increase in SERCA2A mRNA level with a peak at 4 h is found. During prolonged culturing of cells, the preparation progressively develops atrophy (Volz et al., 1991; Pinson et al., 1993) so I could not prolong the time in such a model of experiments. Therefore, I could not determine the subsequent SERCA protein expression and the influence of long-term adrenoceptor stimulation on cell contraction. But the transient expression of SERCA mRNA may suggest, that longer times of adrenergic stimulation would decrease SERCA expression.

This is in agreement with many clinical and experimental observations whereby short-term interventions that improved cardiac contractility were associated with adverse long-term effects on survival and progression of heart failure (Packer et al., 1991; Cohn et al., 1998; O'Connor et al., 1999; Du et al., 2000). β-Adrenoceptor stimulation acutely increases cardiac contractility by the activation of the SERCA2A through protein kinase A-dependent phosphorylation of phospholamban whereas over expression of β_2-adrenergic receptors exacerbates the development of heart failure in mice (Du et al., 2000).

5.4. Limitation of the study

In my study I have used isolated cardiomyocytes for analysis of chronic effects of α- or β-adrenoceptor stimulation on cell function. In this study the cells were kept

quiescent. There was no significant loss of cell protein under these culture conditions and the cells remained rod-shaped. The responses of cells to electrical stimulation were normal. These findings indicate that there is only minor dedifferentiation of cells during the time of experimental analysis. Therefore, in vivo conditions of cell function could be mimicked in this model. However, changes during culturing procedure cannot be totally excluded.

The main advantage of this cell model is to investigate signaling mechanisms induced by only one distinct stimulus i.e. PE or ISO, independent of other cell types and influences in the heart. Thus this cell model is of advantage in regard to the specificity of the investigated agonists and in ruling-out compensatory mechanisms via other neurohumoral factors.

5.5. Conclusion

Several previous studies demonstrate that α- or β-adrenoceptor stimulation can regulate SERCA2A expression. In isolated adult rat cardiomyocytes I have shown that under PE or ISO stimulation decreased cell shortening is found at 0.5 Hz, but not at high beating frequency (2 Hz). Normal cell shortening at high beating frequencies can be maintained by up-regulation of SERCA2A expression, which is mediated via calcineurin/NFAT pathway.

6. Summary

Catecholamines contribute to the adaption of heart to pressure overload via stimulation of adrenoceptors (α- or β-adrenoceptor). While mechanisms resulting in hypertrophic growth of myocytes after α- or β-adrenoceptor-stimulation are established, the functional consequences on cardiomyocyte contractility or hypertrophy are unknown. The present study investigates whether α- or β-adrenoceptor-stimulation by phenylephrine (PE) or isoprenaline (ISO) over 24 h modifies cell shortening in ventricular cardiomyocytes of rat.

In PE-treated myocytes cell shortening at 0.5 Hz was reduced. At 2.0 Hz this reduction was compensated by an increased relaxation velocity. In parallel, SERCA2A expression was increased at protein level and at mRNA level. This induction was independent of PKC activation but dependent on an increase in diastolic calcium. The calcineurin/NFAT pathway was identified, since addition of BAPTA, cyclosporine or NFAT-decoy oligonucleotides reduced SERCA2A expression in presence of PE as regulators of SERCA2A expression. Inhibition of SERCA up regulation under PE by NFAT decoy oligonucleotides reduced cell shortening at high beating frequencies.

Under β-adrenoceptor stimulation similar effects were found: SERCA2A expression was increased under ISO-stimulation and could be reduced to basal level by NFAT decoy oligonucleotides. Decreased cell shortening under ISO is found at 0.5 Hz, but not at high beating frequencies (2 Hz). In myocytes transformed with NFAT decoy oligos decreased cell shortening is present also at 2 Hz under β–adrenergic stimulation.

In conclusion, a functional deficite in contraction of cardiomyocytes due to PE or ISO stimulation can be partially antagonized by PE- or ISO-dependent activation of SERCA expression mediated via the calcium/calcineurin/NFAT pathway.

6.1. Zusammenfassung

Katecholamine tragen über die Aktivierung α- oder β-Adrenorezeptoren Zur Anpassung des Herzens an erhöhte Druckbelastung bei. Während Mechanismen der Hypertrophie-Induktion unter α- oder β–adrenerger Stimulation weitgehend bekannt sind, ist ihr Einfluss auf die kontraktile Funktion von Herzmuskelzellen wenig untersucht. Die hier vorliegende Arbeit untersucht, ob eine 24-stündige Adrenozeptorstimulation mit Phenylephrin (PE) oder Isoprenalin (ISO) die Zellverkürzung in ventrikulären Herzmuskelzellen der Ratte beeinflusst.

In PE-behandelten Herzmuskelzellen kam es zu einer verringerten Zellverkürzung (dL/L), wenn die Zellen mit 0,5 Hz stimuliert wurden. Bei erhöhten Frequenzen (2 Hz) war die Zellverkürzung normal. Dies ging einher mit einer erhöhten Relaxationsgeschwindigkeit und einer Erhöhung der SERCA2A-Expression in PE-behandelten Zellen. Die Induktion der SERCA-Expression war abhängig von einer Erhöhung der diastolischen Kalziumkonzentration. Gabe von BAPTA, Cyclosporin oder NFAT-Decoy-Oligonukleotiden hemmte die PE-induzierte SERCA-Expression, was Kalzium/Calcineurin/NFAT als Regulatoren der SERCA-Expression charakterisiert. Die reduzierte SERCA-Expression nach Transformation mit NFAT-Decoy-Oligonukleotiden hatte eine erniedrigte Zellverkürzung bei elektrischer Stimulation auch unter hohen Frequenzen (2 Hz) zur Folge.

Unter β-adrenerger Expression wurden ähnliche Effekte gefunden: Die SERCA2A-Expression wurde durch Gabe von Isoprenalin erhöht. Diese Induktion konnte durch NFAT-Decoy-Oligonukleotide verhindert werden. Eine reduzierte Zellverkürzung wurde in ISO-behandelten Zellen bei elektrischer Stimulation mit 0,5 Hz, aber nicht mit 2 Hz gefunden. Nach Transformation der Herzmuskelzellen mit NFAT-Decoy-Oligonukleotiden wurde eine reduzierte Zellverkürzung auch bei 2 Hz in ISO-stimulierten Zellen gemessen.

Schlussfolgerung: Ein funktionelles Defizit der kontraktilen Zellverkürzung durch Inkubation von Herzmuskelzellen mit PE oder ISO kann zum Teil durch eine Erhöhung der SERCA-Expression ausgeglichen werden. Die Expression der

SERCA unter PE- oder ISO-Stimulation wird über den Kalzium/Calcineurin/NFAT-Signalweg vermittelt.

7. References

Allo SN, Carl LL and Morgan HE (1992) Acceleration of growth of cultured cardiomyocytes and translocation of protein kinase C. Am J Physiol 263: C319-C325.

Antos CL, McKinsey TA, Frey N, et al. (2002) Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A; 99: 907–12.

Aoyagi T, Yonekura K, Eto Y, Matsumoto A, Yokoyama I, Sugiura S, Momomura S, Hirata Y, Baker DL and Periasamy M (1999) The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) gene promoter activity is decreased in response to severe left ventricular pressure-overload hypertrophy in rat hearts. J Mol Cell Cardiol 31: 919–926.

Arai M, Matsui H and Periasamy M (1994) Sarcoendoplasmic reticulum gene expression in cardiac hypertrophy and heart failure. Circ Res 74: 555–564.

Baker D, Hashimoto K, Grupp I, Ji Y, Reed T, Loukianov E, Grupp G, Bhagwhat A, Hoit B, Walsh R, Marbán E, Periasamy M (1998) Targeted over expression of the sarcoplasmic reticulum Ca^{2+}-ATPase increases cardiac contractility in transgenic mouse hearts. Circ Res 83: 1205–1214.

Bartolome J, Guguenard J, Slotkin TA (1980) Role of ornithine decarboxylase in cardiac growth and hypertrophy. Science 210: 793–794.

Bers DM and Perez-Reyes E (1999) Ca channels in cardiac myocytes: structure and function in Ca^{2+} influx and intracellular Ca^{2+} release. Cardiovasc Res 42: 339–360.

Bogoyevitch MA, Fuller SJ and Sudgen PH (1993) cAMP and protein synthesis in isolated adult rat heart preparations. Am J Physiol 265: C1247-C1257

Boluyt MO, Zheng JS, Younes A, Long X, O`Neill L, Silerman H, Lakatta EG, Crow MT (1997) Rapamycin inhibits alpha 1-adrenergic receptor stimulated cardiac myocyte hypertrophy but not activation of hypertrophy associated genes. Evidence for involvement of p70S6 kinase. Circ Res 81: 176-186.

Bugaisky LB, Gupta M, and Zak R (1992) Cellular and molecular mechanisms of cardiac hypertrophy. The Heart and Cardiovascular System (2^{nd} ed.), edited by H.A. Fozzard New York: Raven 1621-1640.

Bustamante JO, Ruknudin A and Sachs F (1991) Stretch-activated channels in heart cells: relevance to cardiac hypertrophy. J Cardiovasc Pharmacol 17: S110–S113.

Cain BS, Medlum DR, Joo KS, Wang JF, Meng X, Clefeland JC Jr, Banerjee A and Harken AH (1998) Human SERCA2a levels correlate inversely with age in senescent human myocardium. Am J Coll Cardiol 32: 458–467.

Chen F, Ding S, Lee BS and Wetzel GT (2000) Sarcoplasmic reticulum Ca^{2+} ATPase and cell contraction in developing rabbit heart. J Mol Cell Cardiol 32: 745–755.

Chu G, Carr AN, Young KB, Lester JW, Yatani A, Sanbe A, et al. (2002) Enhanced myocyte contractility and Ca^{2+} handling in a calcineurin transgenic model of heart failure. Cardiovasc. Res 54: 105–116.

Clark WA, Rudnick SJ, Simpson DG, LaPres JJ, and Decker RS (1993) Cultured adult cardiac myocytes maintain protein synthetic capacity of intact adult hearts. Am J Physiol 264: H573-H582.

Cohen SS, Polyamines and the animal cells. In: Cohen SS, editor, A guide to the polyamines, New York: Oxford University Press, 1998, pp. 184–203.

Cohn JN, Goldstein SO, Greenberg BH, Lorell BH, Bourge RC, Jaski BE, Gottlieb SO, McGrew F III, DeMets DL, White BG (1998) A dose-dependent increase in mortality with vesnarinone among patients with severe heart failure. N Engl J Med 339:1810–1816.

De Jonge HW, Atsma DE, van der Valk-Kokshoorn EJ, van Heugten HA, van der Laarse A, Lamers JM (1995) Alpha-adrenergic agonist and endothelin induced intracellular Ca^{2+} response in the presence of a Ca^{2+} entry blocker in cultured rat ventricular myocytes. Cell Calcium 18: 515-525.

De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, Braz JC, Glascock BJ, Kimball TF, Del Monte F, Hajjar RJ, and Molkentin JD (2001) Targeted inhibition of calcineurin attenuates cardiac hypertrophy in vivo. Proc Natl Acad Sci USA 98: 3322-3327.

De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW, Kitsis RN and Molkentin JD (2000) Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo. Circ Res 86: 255-263.

Decker RS, Cook MG, Behnke-Barclay MM, Decker ML, Lesch M and Samarel SM (1993) Catecholamines modulate protein turnover in cultured, quiescent rabbit cardiac myocytes. Am J Physiol 265: H329-H339.

Del Monte F, Harding SE, Schmidt U, Matsui T, Kang ZB, Dec GW, Gwathmey JK, Rosenzweig A, Hajjar RJ (1999) Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. Circulation 100: 2308–2311.

Du X-J, Autelitano DJ, Dilley RJ, Wang B, Dart AM, Woodcock EA (2000) β_2-Adrenergic receptor over expression exacerbates development of heart failure after aortic stenosis. Circulation 101: 71–77

Dubus I, Samuel J-L, Marotte F, Delcayre C and Rappaport L (1990) β-Adrenergic agonist stimulate the synthesis of noncontractile but not contractile proteins in cultured myocytes isolated from adult rat heart. Circ Res 66: 867-874.

Dumas AR, Wisnewsky C, Boheler KR, Keurs HT, Fiszman MY and Schwartz K (1997) The sarco(endo)plasmic reticulum Ca^{2+}-ATPase gene is regulated at the transcriptional level during compensated left ventricular hypertrophy in the rat. Comptes Rendus de l'Academie des Sciences-Series III-Sciences de la Vie. 320: 963–969.

Fabiato A (1983) Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol 245: C1–C14.

Feldman AM, Weinberg EO, Ray PE and Lorell BH (1993) Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding. Circ Res 73: 184–192.

Fisher DJ, Tate CA and Phillips S (1992) Developmental regulation of the sarcoplasmic reticulum pump in the rabbit heart. Pediatr Res 31: 474–479.

Fuller SJ, Gaitanaki CJ, and Sugden PH (1989) Effects of increasing extracellular pH on protein synthesis and protein degradation in the perfused working rat heart. Biochem J 259: 173-179.

Fuller SJ, Gaitanaki CJ, and Sugden PH (1990) Effects of catecholamines on protein synthesis in cardiac myocytes and perfused hearts isolated from adult rats. Biochem J 266: 727-736.

Fuller SJ, Gaitanaki CJ, Hatchett RJ and Sugden PH (1991) Acute α_1-adrenergic stimulation of cardiac protein synthesis may involve increased intracellular pH and protein kinase activity. Biochem J 273: 347-353.

Giordano FJ, He H, McDonough P, Meyer M, Sayen MR, Dillmann WH (1997) Adenovirus-mediated gene transfer reconstitutes depressed sarcoplasmic reticulum Ca^{2+}-ATPase levels and shortens prolonged cardiac myocyte Ca^{2+} transients. Circulation 96: 400–403.

Gombosova I, Boknik P, Kirchhefer U, Knapp J, Luss H, Muller FU, Muller T, Vahlensiek U, Schmitz W, Bodor GS and Neumann J (1998) Postnatal changes in contractile time parameters, calcium regulatory proteins, and phosphatases. Am J Physiol 274: H2123–H2132.

Hajjar RJ, Schmidt U Matsui T, Guerrero JL, Lee KH, Gwathmey JK, Dec GW, Semigeran MJ, Rosenzweig A (1998) Modulation of ventricular function through gene transfer in vivo. Proc Natl Acad Sci USA 95: 5251–5256.

He H, Giordano FJ, Hilal-Dandan R, Choi D-J, Rockman HA, McDonough PM, Bluhm WF, Meyer M, Sayen MR, Swanson E, Dillmann WH (1997) Over expression of the rat sarcoplasmic reticulum Ca^{2+} ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. J Clin Invest 100: 380–389.

Hongo K, White E, Gannier F, Argibay JA Garnier D and Orchard CH (1995) Effect of stretch on contraction and the Ca^{2+} transient ferret ventricular muscles during hypoxia and acidosis. Am J Physiol 269: C690–C697.

Houser SR, Piacentino V and Weisser J (2000) Abnormalities of calcium cycling in the hypertrophied and failing heart. J Mol Cell Cardiol 32: 1595–1607.

Igarashi K, Kakegawa T, Hirose S (1982) Stabilization of 30 S ribosomal subunits of Bacillus subtilis W168 by spermidine and magnesium ions. Biochim Biophys Acta 755:326–331.

Ito K, Yan X, Feng X, Manning WJ, Dillman WH, Lorell BH (2001) Transgenic expression of sarcoplasmic reticulum Ca^{2+} ATPase modifies the transition from hypertrophy to early heart failure. Circ Res 89: 422-429.

Izumi Y, Kim S, Zhan Y, Namba M, Yasumoto H, and Iwao H (2000) Important role of angiotensin II-mediated c-Jun NH_2-terminal kinase activation in cardiac hypertrophy in hypertensive rats. Hypertension 36: 511–516.

Jain J, McCaffrey PG, Valge-Archer VE and Rao A (1992) Nuclear factor of activated T cells contains Fos and Jun. Nature 356: 801-804.

Karliner JS, Kariya T and Simpson PC (1990) Effects of pertussis toxin on α_1-agonist-mediated phosphatidylinositide turnover and myocardial cell hypertrophy in neonatal rat myocytes. Experientia 46: 81–84.

Kiss E, Ball NA, Kranias EG and Walsh RA (1995) Differential changes in cardiac phospholamban and sarcoplasmic reticular Ca^{2+}-ATPase protein levels. Effects on Ca^{2+}transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure. Circ Res 77: 759–764.

Leite MF, Page E and Ambler S K (1994) Regulation of ANP secretion by endothelin-1 in cultured atrial myocytes: desensitization and receptor subtype. Am J Physiol 267: H2193–H2203.

Levy D, Garrison RJ, Savage D, Kannel WB, Castelli WP (1990) Prognostic implication of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Eng J Med 322: 1561-1566.

Luss I, Boknik P, Jones LR, Kirchhefer U, Knapp J, Linck B, Luss H, Meissner A, Muller FR, Schmitz W, Vahlensieck U and Neumann J (1999) Expression of cardiac calcium regulatory proteins in atrium v ventricle in different species. J Mol Cell Cardiol 31: 1299–1314.

Fernando M, Garcia-Rodriguez C and Rao A (2000) Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. EMBO 19: 4783-4795.

MacLennan DH (1970) Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J Biol Chem 245: 4508–4518.

Marban E, Kitakaze M, Kusuoka H, Porterfield JK, Yue DT and Chacko VP (1987) Intracellular free calcium concentrations measured with 19F NMR spectroscopy in intact ferret hearts. Proc Natl Acad Sci USA 84: 6005–6009.

Matsui H, MacLennan DH, Alpert N and Periasamy M (1995) Sarcoplasmic reticulum gene expression in pressure overload-induced cardiac hypertrophy in rabbit. Am J Physiol 268: C252–C258.

Meyer M, and Dillmann WH (1998) Sarcoplasmic reticulum Ca^{2+}-ATPase over expression by adenovirus mediated gene transfer and in transgenic mice. Cardiovasc Res 37: 360–366.

Miyamoto MI, Del Monte F, Schmidt U, DiSalvo TS, Kang ZB, Matsui T, Guerrero JL, Gwathmey JK, Rosenzweig A, Hajjar RJ (2000) Adenoviral gene transfer of

SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. Proc Natl Acad Sci USA 97: 793–798.

Mochly-Rosen D, Wu G, Hahn H, Osinska H, Liron T (2000) Cardiotrophic effect of εprotein kinase C: analysis by in vivo modulation of εPKC translocation. Circ Res 86: 1173-1179.

Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR and Olson EN (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93: 215-228.

Molkentin JF (2004) Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. Cardiovasc Res 63: 467-475

Morgan HE, and Baker KM (1991) Cardiac hypertrophy: mechanical, neural and endocrine dependance. Circulation 83: 13-25.

Muller OJ, Lange M, Rattunde H, Lorenzen HP, Muller M, Frey N, et al. (2003) Transgenic rat hearts overexpressing SERCA2a show improved contractility under baseline conditions and pressure overload. Cardiovasc Res 59: 380–389

Nagai R, Herzberg AZ, Brandl CJ, Fuji J, Tada M, MacLennan DH, Alpert NR and Periasamy M (1989) Regulation of myocardial Ca^{2+}ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone. Proc Natl Acad Sci USA 86: 2966–2970.

O'Connell TD, Ishizaka S, Nakamura S, Swigart PM, Rodrigo MC, Simpson GL, Cotecchia S, Rokosh DG, Grossman W, Foster E & Simpson PC (2003) The alpha(1A/C)- and alpha(1B)-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. J Clin Invest 111: 1783-1791.

O'Connor CM, Gattis WA, Uretsky BF, Adams KF Jr, McNulty SE, Grossman SH, McKenna WJ, Zannad F, Swedberg K, Gheorghiade M, Califf RM (1999) Continuous intravenous dobutamine is associated with an increased risk of death in patients with advanced heart failure: insights from the Flolan International Randomized Survival Trial (FIRST). Am Heart J 138: 78–86.

Omura T, Yoshiyama M, Yoshida K, Nakamura Y, Kim S, Iwao H, Takeuchi K, and Yoshikawa J (2002) Dominant negative mutant of c-Jun inhibits cardiomyocyte hypertrophy induced by endothelin-1 and phenylephrine. Hypertension 39: 81–86.

Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, Hendrix GH, Bommer WJ, Elkayam U, Kukin ML, Mallis GI, Sollano JA, Shannon J, Tandon PK, DeMets DL (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. N Engl J Med 325: 1468–1475.

Parsons SA, Millay DP, Wilkins BJ, Bueno OF, Tsika GL, Neilson JR, Liberatore CM, Yutzey KE, Crabtree GR, Tsika RW and Molkentin JD (2004) Genetic loss of calcineurin blocks mechanical overload-induced skeletal muscle fiber type switching but not hypertrophy. J Biol Chem 279: 26192-26200.

Periasamy M and Huke S (2001) SERCA pump level is a critical determinant of Ca^{2+} homeostasis and cardiac contractility. J Mol Cell Cardiol 33: 1053-41063.

Pinson A, Schlüter K-D, Zhou XJ, Schwartz P, Kessler-Icekson G and Piper HM (1993) Alpha- and beta-adrenergic stimulation of protein synthesis in cultured adult ventricular cardiomyocytes. J Mol Cell Cardiol 25: 477-490.

Pönicke K, Schlüter K-D, Heinroth-Hoffmann I, Seyfarth T, Goldberg M, Osten B, Piper HM and Brodde O-E (2001) Noradrenaline-induced increase in protein synthesis in adult ventricular cardiomyocytes: involvement of only α_{1A}-adrenoceptors. Naunyn-Schmiedeberg's Arch Pharmacol 364: 444-453.

Pu WT, Ma Q, Izumo S (2003) NFAT transcription factors are critical survival factors that inhibit cardiomyocytes apoptosis during phenylephrine stimulation in vitro. Circ Res 92 725-731.

Qi M, Shannon TR, Euler DE, Bers DM and Samarel AM (1997) Downregulation of sarcoplasmic reticulum Ca^{2+}-ATPase during progression of left ventricular hypertrophy. Am J Physiol 272: H2416–H2424.

Rao A, Luo C and Hogan PG (1997) Transcription factor of the NFAT family: regulation and function. Annu Rev Immunol 15: 707-747.

Rapacciulo A, Esposito G, Caron K, Mao L, Thomas SA and Rockman HA (2001) Important role of endogenous norepinephrine and epinephrine in the development of in vivo pressure-overload cardiac hypertrophy. J Am Cell Cardiol 38: 876-882.

Reed TD, Babu GJ, Ji Y, Zilberman A, Ver Heyen M, Wuytack F and Periasamy M (2000) The expression of SR calcium transport ATPase and the Na/Ca exchanger are antithetically regulated during mouse cardiac development and in hypo/hyperthyroidism. J Mol Cell Cardiol 32: 453–464.

Van Rooij E, Doevendans PA, de Theije CC, Babiker FA, Molkentin JD, and De Windt LJ (2002) Requirement of nuclear factor of activated T-cells in calcineurin-mediated cardiomyocyte hypertrophy. J Biol Chem 277: 48617-48626.

Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN and Williams RS (2001) Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc Natl Acad Sci USA 98: 3328-3333.

Sadoshima J, and Izumo S (1993) Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocytes in vitro. Circ Res 73: 424–438.

Sadoshima J, Xu Y, Slayter HS and Izumo S (1993) Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 75: 977–984.

Sanbe A, Gulick J, Hanks MC, Liang Q, Osinska H, Robbins J (2003) Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. Circ Res 92: 609–616.

Schäfer M, Schäfer C, Piper HM and Schlüter K-D (2002) Hypertrophic responsiveness of cardiomyocytes to α- or β-adrenoceptor stimulation requires sodium-proton-exchanger-1 (NHE-1) activation but not cellular alkalization. Eur J Heart Failure 4: 249-254.

Schlüter K-D, Zhou XJ and Piper HM (1995). Induction of hypertrophic responsiveness to isoproterenol by TGF-beta in adult rat cardiomyocytes. Am J Physiol 269: C1311–C1316.

Schlüter K-D, Simm A, Schäfer M, Taimor G, Piper HM (1999) Early response kinase and PI 3-kinase activation in adult cardiomyocytes and their role in hypertrophy. Am J Physiol 276: H1655-1663.

Schlüter K-D, Millar BC, McDermott BJ and Piper HM (1995) Regulation of protein synthesis and degradation in adult ventricular cardiomyocytes. Am J Physiol 269 (Cell Physiol. 38): C1347-C1355.

Schlüter K-D, Schäfer M, Balser C, Taimor G, Piper HM (1998) Influence of pHi and creatine phosphate on α-adrenoceptor mediated cardiac hypertrophy. J Mol Cell Cardiol 30: 763-771.

Schlüter K-D and Wollert K (2004) Synchronization and integration of multiple hypertrophic pathways in the heart. Cardiovasc Res 63: 367-372.

Schlüter K-D, Goldberg Y, Taimor G, Schäfer M and Piper HM (1998) Role of phosphatidylinositol 3-kinase activation in the hypertrophic growth of adult ventricular cardiomyocytes. Cardiovasc Res 40: 174-181.

Schlüter K-D and Piper HM (1992) Trophic effect of catecholamines and parathyroid hormone on adult ventricular cardiomyocytes. Am J Physiol 263 (Heart Circ Physiol 32): H1739-H1746.

Simm A, Schlüter K-D, Diez C, Piper HM and Hoppe J (1998) Activation of $p70^{s6}$ kinase by β-adrenoceptor agonists on adult cardiomyocytes. J Mol Cell Cardiol 30: 2059-2067.

Simmermann HK and Jones LR (1998) Phospholamban: protein structure, mechanism of action, and role in cardiac function. Physiol Rev 78: 921–947.

Stengl M, Mubagwa K, Carmeliet E and Flameng W (1998) Phenylephrine-induced stimulation of Na+/Ca2+ exchange in rat ventricular myocytes. Cardiovasc Res 38: 703-710.

Taffet GE, Pham TT, Bick DL, Entman ML, Pownall HJ and Bick RJ (1993) The calcium uptake of the rat heart sarcoplasmic reticulum is altered by dietary lipid. J Membr Biol 131: 963–998.

Taimor G, Schlüter K-D, Best P, Helmig S and Piper HM (2004) Transcription activator protein 1 mediates α- but not β-adrenergic hypertrophic growth responses in adult ventricular cardiomyocytes. Am J Physiol 286: H2369-H2375.

Takemoto Y, Yoshiyama M, Takeuchi K, Omura T, Komatsu R, Izumi Y, Kim S, and Yoshikawa J (1999) Increased JNK, AP-1 and NF-κB DNA binding activities in isoproterenol-induced cardiac remodeling. J Mol Cell Cardiol 31: 2017–2030.

Takizawa T, Arai M, Yoguchi A, Tomaru K, Kurabayashi M and Nagai R (1999) Transcription of the SERCA2 gene is decreased in pressure-overloaded hearts: a study using in vivo direct gene transfer into living myocardium. J Mol Cell Cardiol 31: 2167–2174.

Van Leeuwen JE and Samelson LE (1999) T-cell antigen-receptor signal transduction. Curr Opin Immunol 1: 242-248.

Vlasblom R, Muller A, Musters RJP, Zuidwijk MJ, van Hardeveld C, Paulus WJ, Simonides WS (2004) Contractile arrest reveals calcium-dependent stimulation of SERCA2a mRNA expression in cultured ventricular cardiomyocytes. Cardiovasc Res 63: 537-544.

Volz A, Piper HM, Siegmund B, Schwartz P (1991) Longevity of adult ventricular rat heart muscle cells in serum-free primary culture. J Mol Cell Cardiol 23: 161-173.

Yano M, Kim S, Izumi Y, Yamanaka S, and Iwao H (1998) Differential activation of cardiac c-jun amino-terminal kinase and extracellular signal-regulated kinase in angiotensin II-mediated hypertension. Circ Res 83: 752–760.

Zobel C, Kassiri Z, Nguyen TT, Meng Y and Backx PH (2002) Prevention of hypertrophy by over expression of Kv4.2 in cultured neonatal cardiomyocytes. Circulation 106: 2385-2391.

Zou Y, Hiroi Y, Uozumi H, Takimoto E, Toko H, Zhu W, Kudoh S, Mizukami M, Shimoyama M, Shibasaki F, Nagai R, Yazaki Y, and Komuro I (2001) Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. Circulation 104: 97-101.

8. Ph.D Published Data

Publication

Attia Anwar, Gerhild Taimor, Hüdayi Korkususz, Rolf Schreckenberg, Tobias Berndt, Yasar Abdallah, Hans Michael Piper, Klaus-Dieter Schlüter. (2005) PKC-independent signal transduction pathway increase SERCA2 expression in adult rat cardiomycytes. J Mol Cell Cardiol. 39, 911-919

Presentations

1. **A. Anwar**, K. D. Schlüter, T. Berndt, M. M. Anwar, G. Euler. (2006). β-adrenerge Stimulation erhöht über NFAT-vermittelt SERCA-Expression in ventrikulären Herzmuskelzellen. Deutsche Gesellschaft für Kardiologie Herz und Kreislaufforschung e.V. 72.Jahrestagung, Congress Centrum Mannheim.

2. M. M. Anwar, J. Heger, **A. Anwar**, G. Euler. (2006) Pro- und anti-apoptotische Effekte von Mitgliedern der TGF β-Superfamilie auf ventrikuläre Herzmuskelzellen der Ratte. Deutsche Gesellschaft für Kardiologie Herz und Kreislaufforschung e.V. 72.Jahrestagung, Congress Centrum Mannheim.

3. **A. Anwar**, M. M. Anwar, K. D. Schlüter, H. M. Piper, G. Euler. (2006) NFAT mediated SERCA upregulation under β-adrenoceptor stimulation in ventricular cardiomyocytes of rat. Acta Physiologica, 186, suppl. 1, PM06A-5.

4. M. M. Anwar, J. Heger, **A. Anwar**, H. M. Piper, G. Euler. (2006) Pro and anti-apoptotic effects of TGFβ-superfamily members in cardiomyocytes. Acta physiologica, 186, Suppl. 1, PM06A-9.

5. **A. Anwar**, K. - D. Schlüter, R. Schreckenberg, H. Korkusuz, T. Berndt, Y. Abdallah, H. M. Piper and G. Taimor. (2005) Increased SERCA expression after α-adrenergic stimulation and functional consequences in cardiomyocytes. European Journal of Physiology, 449, Suppl. 1, S31.

6. M. M. Anwar, J. Heger, **A. Anwar**, H. M. Piper and G. Taimor. (2005) The TGFβ-superfamily and apoptosis induction in cardiomyocytes. European Journal of Physiology, 449, Suppl. 1, S31.

7. K. D. Schlüter, G. Taimor, R. Schreckenberg, T. Berndt, H. M. Piper, Y. Abdullah, H. Korkusuz, **A. Muqaddas**. (2004) Impact of catecholamines on SERCA2A expression: signal transduction and funtional consequences. European Heart Journal, 25, Suppl., 178, P1110.

9. Curriculum Vitae

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EDUCATIONAL PROFILE

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Thesis topic	Genome variation study in well characterised chronic HBV isolates of Pakistan
University	National Centre of Excellence in Molecular Biology University of Punjab Lahore Pakistan.

M.Phil Published data

Zafar,I**., Attia,M**., Naaz,A., Idrees,M. and Riazuddin,S. Hepatitis B virus isolate ZAFAR41A large S protein gene, partial cds. AF388661

Year	1998-1999
Degree	Bachelor of Education (B.Ed.)
Subject	Chemistry & Biology
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MSc Publication

1. **Attia M**, Zafar I and Tanveer A. Studies on optimum growth conditions and antibiotic resistance of bacterial flora isolated from freshwater lymnaeid snail. Acta Sci. 1999,9(1): 89-102.

2. Zafer I, **Attia M** and Tanveer A. Some studies on bacteria haboured by freshwater physid snails in natural conditions. Acta Sci. 1999,9(1):103-116.

MSc Abstract

1 **Attia M**, Zafar I and Tanveer A. Biological control of fascioliasis vector snail using bacteria as biological control agents. ISC. 2000, 2:5.

2. Zafar I, **Attia M** and Tanveer A. Isolation of freshwater snail and its evaluation as biomolluscicide.ISC. 2000, 2:6.

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11. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Attia Anwar

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